Physicochemical properties of wheat starches and their relationship to liquefaction and fermentative bioethanol performance.

by

Jessica Saunders

A thesis presented to the University of Manitoba
In fulfillment of the
Thesis requirement for the degree of
Master of Science
In
Biosystems Engineering
Winnipeg, Manitoba, Canada

Copyright © 2010 by Jessica Saunders

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Fourteen varieties of wheat, and one sample each of triticale and corn, grown in western Canada were assessed for differences in starch content and structure and their susceptibility to industrial α-amylase once gelatinzied. Starch content was found to vary significantly between samples and correlated strongly with protein content ($r=-0.895$, $P<0.001$). AC Andrew, a soft white spring (SWS) wheat, possessed high starch (68.6±0.09%) and low protein (10.1%) characteristics more similar to Central Manitoba Corn (CMC) than other measured wheat cultivars. Physicochemical properties of starch such as amylopectin to amylose ratio, starch granule morphology, and thermal and pasting properties were all found to vary significantly between varieties. The branch chain length distribution of amylopectin was not found to be significantly different between samples. Amylopectin to amylose ratio for wheat starch ranged from 73.60:26.40 to 69.47:30.53 and was found to be different and lower than CMC at 74.51:25.49. Enzymatic susceptibility was measured using industrial α-amylase to hydrolyze gelatinized starches and resultant reducing sugar content ranged from approx. 407 to 500mg glucose equivalents per gram starch, indicating different patterns of oligosaccharide chain lengths present after hydrolysis. However, liquefaction performance was measured and found to be similar between samples, indicating no major differences in the quantities of maltose, maltotriose, maltotetrose or larger dextran concentrations in the respective worts prior to saccharification and fermentation. During fermentation striking differences in glucose generation were observed, the high glucose cohort averaged 1.21 g/g-starch for the initial time point, compared to a range of 0.83 to 1.05 g/g-starch for the low glucose cohort. In general, the pattern of glucose generation appears to be consistent with ethanol and

biomass production. CMC and Buteo, observed to have the highest ethanol and biomass yields, achieved conversion efficiencies between ~95.6-97%, compared to the low glucose cohort which achieved 85.64-88.42% conversion efficiency. Amylose content is observed in this study to have a moderate negative correlation with ethanol yield ($r=-0.784$, $P<0.05$), suggesting even subtle decreases in wheat starch amylose content (~4%) correspond to increases in generated ethanol. Reducing sugar yield also showed a strong negative correlation ($r=-0.921$, $P<0.01$) with ethanol yield. Reducing sugar yield, however, appears to be influenced by amylopectin to amylose ratio. Correlating structural attributes with fermentation performance suggests that amylopectin to amylose ratio is the most predictive factor in the pattern of wheat starch hydrolysis.

Acknowledgements

I would like to sincerely thank the many individuals who have aided in the preparation of this thesis, as I would never have been able to undertake a project of such scope without your support. I would like to acknowledge the efforts of Dr. David Levin and Dr. Marta Izydorczyk, who have both been tremendous teachers and allies over the course of my degree. In particular, Dr. Levin has been an invaluable source of support and encouragement, whose inextinguishable enthusiasm and unique vision have made so much possible. For her technical expertise and tutilage, I would like to thank Dr. Izydorcczyk. I have the deepest respect for your methods, Marta, and it has been a true pleasure to work with someone as thorough and dedicated to their subject. To everyone at the Canadian Grain Commission who offered their time and patience in helping with the myriad tasks presented to me in the coarse of my research, I thank you heartily. The efforts of Sharon Bazin and Tricia McMillan, in particular, have been immeasurably helpful. A special thanks to Dr. Anita Brule Babel, for provisioning both the raw materials necessary for the project and sage counsel whenever it was needed. Lastly, I would like to thank Dr. Nazim Cicek and Dr. Richard Sparling, who happily offered their thoughts and resources to help solve innumerable problems encountered during experimentation.

I'd like to thank all my friends and colleagues at the University of Manitoba, without whom I would never have completed this degree. To Debby Watson and Evelyn Fehr, thank you so much for your patience, you deserve all the gratitude I have to offer.

This work was supported by funds provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Husky Energy.

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

Bioethanol is currently the most widely used liquid biofuel in the world. Global ethanol production was ~19 billion L in 2000, and production has almost quadrupled over the past decade, with estimates for 2012 approaching 76 billion L (MRA 2008). At present, bioethanol is produced exclusively via 1st generation technologies, utilizing sugar and starch rich feedstock, as no 2nd generation commercial size cellulosic ethanol facilities are presently in operation. As nation's move toward increasing incorporation of bioethanol into their respective transportation fuel supplies, it is likely that starch-based technologies will play a growing role, at least in the near future, in fulfilling that demand. Despite the great potential of cellulosic technologies to offset petroleum consumption in the future, the present reliance on starch-based bioethanol indicates the continued need for process improvement and feedstock refinement of the 1st generation production scenario, ensuring bioethanol is as cost effective as possible in the short-term.

The mandate for incorporation of starch-based biofuels into the transportation economies of many countries, such as the United States of America (U.S.), the European Union (EU), and Canada, is not yet economically viable, as the bioethanol industry does not function, as of yet, without government subsidies. Rather, the production of biofuels from what have traditionally been food crops, such as wheat and corn, addresses a myriad of pressing concern over climate change, diversification of energy supply, and development of local economies. Even when considering the probable economic parity of starch-based ethanol with petroleum, it is still unlikely that starch-based ethanol production has the potential to singularly address long-term, global transportation fuel demand. The myriad of issues related to food diversion, land use patterns, and limited

supply make starch-based ethanol production an imperfect solution whose utility is best viewed as a bridge towards a future necessarily reliant on diverse and locally determined energy strategies. In countries where cereal grains are locally available and abundant, the dedication of certain lands to high yielding bioenergy crops could feasibly contribute to local transportation fuel demand without disrupting the food supply. In order for starch-based bioethanol to contribute maximally to society's energy needs grains possessing optimized characteristics for end-use as bioethanol feedstock are indicated for development. Development of wheat and corn cultivars, with characteristics that are ideally suited for bioethanol production, will therefore ensure access to stable supply, reduce competition for feed and food versus fuel, and increase the economic viability of grain-based ethanol production.

Grain characteristics tailored to the needs of the bioethanol industry include compositionally high contents of starch and physicochemical parameters of starch lending themselves to high conversion efficiencies under industrial conditions. However, no evaluative criteria of grain starch quality, as it relates to maximized ethanol yield, is available, as these relationship are not well understood. Starch with high intrinsic resistance to enzymatic hydrolysis, although of possible high compositional content, could yield a low sugar load to yeast, making it an erroneous selection as feedstock, especially when considering the industries move towards ever higher starch feed streams. Of particular interest to the bioethanol industry are the functional properties of starch lending themselves to ease of amylolytic hydrolysis and high conversion efficiency of starch to fermentable sugar. This study, then, attempts to elucidate the key physicochemical parameters of western Canadian wheat starch bearing influence on fermentative bioethanol yield, with the ultimate aim of assisting producers in selecting grain well suited

to bioethanol end-use. In the present study, select cultivars from 6 of the 8 milling classes of wheat in western Canada, as well as wheat from the Eastern Canada General Purpose (ECGP) class, were examined for potential use as fuel ethanol feedstock.

Chapter 2: Literature Review

2.1 Ethanol's Role in the World's Fuel Economy

2.1.1 Drivers for the Development of an Ethanol Economy

Biofuels, such as ethanol and bio-diesel, are poised to serve as not only an agent of greenhouse gas reduction but also as a means to secure an energy supply that is local, renewable and independent of a financially volatile and potentially unreliable oil market. For example, of the roughly 20 million barrels of crude oil the U.S. consumes daily (EIA 2009b) almost 60% is imported (EIA 2009a), making the U.S. highly vulnerable to oil market fluctuations. The role of ethanol as a blended gasoline product, in ratios of 5 – 20% (v/v) ethanol, referred to as gasohol, has many advantages when considering the ease of adaptation to present oil infrastructure. Alcohol blended gasoline can be used without major modifications to pre-existing automobile engines and burns cleaner, due to the higher octane rating, reducing harmful emissions (Agarwal 2007). As gasoline prices increase and emission regulations become more stringent, ethanol is likely to assume a meaningful role in a market that no longer has access to cheap and abundant petroleum products.

Ethanol generation from biomass is but one alternative energy scheme aimed at reducing global CO_2 emissions by displacing consumption of fossil fuel. Fossil fuel combustion is the single largest human influence on climate, accounting for 80% of anthropogenic greenhouse gas emissions (Quadrelli and Peterson 2007). The rate of CO_2 emissions has been steadily increasing over the last 100 years. During the pre-industrialized era global CO_2 levels were relatively steady at about 280 parts per million (ppm). The 2004 concentration of CO_2 (377 ppm) was about 35% higher than a century

and a half ago, with the fastest growth occurring in the most recent years; 1.8 ppm/year in the period 1999-2004 (Quadrelli and Peterson 2007). If the present level of emissions remains unabated the global concentration of atmospheric CO_2 for the year 2100 is projected to range from 540 – 970 ppm (Trenberth 2007). The correlation between atmospheric CO_2 concentration and global temperature could cause a purported rise of 1.4 – 5.8 ^{o}C in global average surface temperature over the next 100 years; representing an unprecedented rate of global temperature change over the last 10,000 years. Global warming of more than approximately 1 ^{o}C, relative to 2000, will constitute "dangerous" climate change as judged from likely effects on sea level and extermination of species (Hansen et al. 2006). In order to control anthropogenic greenhouse gas emissions, thus stabilizing atmospheric CO_2 levels, non-fossil fuel based energies are necessarily being explored with increasing interest.

Fossil fuels at present provide 85% of the commercial energy consumed worldwide (Lackner and Sachs 2005) and 40% of the total energy consumption in the world is in the form of liquid fuels (Tan et al. 2008). According to an estimate, the reserves will last for 218 years for coal, 41 years for oil, and 63 years for natural gas, under a business as usual scenario (Agarwal 2007). The world's supply of cheap crude oil is dwindling, with 'peak oil' having occurred or likely to occur before 2010 (Lackner and Sachs 2005) As the population swells towards 9 billion it is estimated that oil demand will double in the pre-industrialized countries of China and India, 'making global oil demand rise by 52% by 2025' (IEA 2005). Projections for the 30-year period from 1990-2020 indicate that vehicle travel, and consequently fossil-fuel demand, will almost triple (Agarwal 2007). The rising demand for fossil fuels coupled with a dwindling supply

creates a context where oil prices are likely to soar. As oil reserves become scant and therefore expensive, alternative energy schemes become increasingly competitive.

The present drivers for the development of the world's biofuel economy have little to do with economic gain, as these industries do not, as of yet, exist without heavy government subsidies. Subsidies per liter of ethanol are 60 times greater than the subsidies per liter of gasoline (Pimentel et al. 2009) and total more than $6 billion per year for U.S. corn ethanol (Koplow 2006). Many governments have mandated the incorporation of biofuels into their transportation economies to address global greenhouse gas emissions, fuel security, volatility in oil pricing and the development of local, rural economies. In order to achieve true market viability, however, ethanol technologies must assume economic parity with other energy schemes. Much research is presently directed at ways to improve conversion technologies, exploring technical and economic biomass potentials and possible environmental impacts (Slingerland and van Geuns 2005). If ethanol is to have an impact on society's long-term energy needs, technical advancements must be made to improve both environmental and economic aspects of production.

2.1.2 The Economics of Ethanol

The largest ethanol producing industries, representing over 90% of the global 65.7 billion L produced in 2008 (RFA 2008), are located in Brazil and the U.S. Between 2000 and 2007, the average annual growth rate of U.S. ethanol production was about 23% and in 2006, the U.S. overtook Brazil to become the world's largest ethanol producer (Pryor 2009). Total production capacity in the U.S. is expected to reach about 90% of the 2015 goal of 56.2 billion L of corn ethanol production set in the 2007 Energy Independence and Security Act (Pryor 2009). In 2008 the U.S. produced 34 billion L (Table 2.1) of ethanol for use as fuel oxygenate in blend

Table 2.1. Global ethanol production. Adapted from RFA (2008).

Country	Billions of Liters
USA	34.1
Brazil	24.5
European Union	2.8
China	1.9
Canada	0.9

percentages of primarily 10% (E10) (Tao and Aden 2009). Brazil produced approximately 24.5 billion L in 2008 but mandates 20-25% (E20-E25) blend ratios (Hahn 2008). According to Hahn (2008) the EU has also set targets of 5.75% for blended gas, as has Argentina (at least 5%). China and India are also following this trend with Nation wide fuel ethanol programs (Bai et al. 2008). According to the Canadian Renewable Fuels Association (CRFA) (2009) Canada produced approximately 1.39 billion L/year of ethanol as of January 2009 drawing upon wheat, corn, wood waste, municipal landfill waste and lignocellulosic straws from wheat, barley and oats. Canada will need to produce 2 billion L of ethanol by 2010, however, to meet the 5% federal renewable fuel standard recently mandated (CRFA 2009).

Biofuels, or any other petroleum alternative, are not poised to contribute to energy supply if the price of oil is ≤ $20 (USD) a barrel, as it has been for most of the past three decades (Dale 2008). Dale (2008) claims that at ≥ $50 per barrel of oil, many alternatives make economic sense, including some biofuels and particularly cellulosic ethanol. Most commercially made ethanol now comes from corn (~97.5% in the U.S.) (Zhao et al. 2009) and the most commonly available blended gasoline contains 10% corn ethanol (Pryor 2009). According to Pryor (2009), who summarized the findings of several studies on the performance of ethanol/gasoline blends compared to regular gasoline, it is suggested that

for E10 mileage per gallon is not greatly different than unblended gasoline. For E20 and higher, however, notable declines in fuel economy are observed. For the U.S. during 2006/2007, net ethanol production costs of approximately $\$400/m^3$ (~ \$0.396/L) were documented for corn purchased at \$3.35 per bushel (Tao and Aden 2009). Corn or cereal feedstock is the largest single cost contributor, ranging from 60 to 80% of total production cost. Average corn prices from 2002 to 2008 have ranged from \$2 per bushel to \$4.20 (USDA 2008), cost data suggests that a 10% price increase in corn alone would raise the production cost by 5.9% (Pryor 2009).

Production cost is not the whole cost, however, and considering the larger picture for corn ethanol production, including capital depreciation, markup, shipping and storing, and subsidies, the cost swells to \$1.819/gal (~\$0.48/L) when considering the 20006/2007 baseline (Pryor 2009). Without subsidies, at corn prices of around \$3.25 per bushel, ethanol as a high-octane fuel is competitive with oil at about \$60 per barrel (Dale 2008). The retail price of gasoline in 2009 (Jan.–Oct.) has averaged \$2.486/gal (~\$0.656/L) with crude oil averaging \$58.43/barrel (EIA 2009c), the U.S. average rack price of ethanol in November, 2009 is listed as \$2.2103/gal (~\$0.584/L) (Fuel Ethanol 2009). Under the present pricing scenario, if E10 gasoline has equivalent fuel economy, the incorporation of ethanol as E10 into the fuel supply has no negative effect on fuel price. In fact, the growth in ethanol production, according to an Iowa State University study, has caused retail gasoline prices to be \$0.29 to \$0.40 per gallon lower than would otherwise have been the case (Du and Hayes 2008).

Wheat based ethanol production, which predominates in the EU, has less favorable economics than corn based production. In the EU grain-based ethanol production cost was reported in 2006/2007 as \$1.54/gal (~ \$0.578/L) (Tao and Aden 2009). Wheat has

numerous disadvantages as compared to corn for use as ethanol feedstock. Corn has historically been a cheaper commodity, costing roughly half that of wheat per unit weight. Average corn yields are just below 8600 kg/ha (Patzek 2004) compared to the global average yield of wheat per hectare of 2700 kg/ha (Koutinas et al. 2004). Wheat is 10-15% lower in starch content than corn, which results in reduced concentrations of ethanol in the beer and substantially lower ethanol yields per unit weight (Sosulski and Sosulski 1994). According to Koutinas et al. (2004), bioethanol production from wheat, for a standard volume of ethanol produced, costs roughly 1.6 times that of corn. Feedstock cost is the most substantial component of wheat based ethanol production and accounts for over 60% of total cost (Sosulski and Sosulski 1994). Also, wheat bran, germ, and outer endosperm proteins of the wheat, which do not contribute to the ethanol yield, are carried through the process, further increasing the production cost (Sosulski and Sosulski 1994). Sugarcane, conversely, has more favorable economics than corn based production. Tao and Aden (2009) suggest that Brazilian sugarcane ethanol costs anywhere from $1.14 - $1.29/gal (~$0.30 - $0.34/L), making it cheaper than both corn and grain based production. Sugarcane ethanol in Brazil is reported to offer higher energy return and greenhouse gas reductions per litre of ethanol than US-made corn ethanol (Rajagopal et al. 2007). Regional variability in agricultural conditions dictate the fuel crops that can feasibly be produced in the area. Sugarcane, for example, does not grow outside of temperate or tropical climates.

Transportation biofuels such as cellulosic ethanol, if produced from low-input biomass grown on agriculturally marginal land or from waste biomass, could provide much greater supplies and environmental benefits than food-based biofuels (Hill et al. 2006). The US government, under the Energy Independence and Security Act of 2007,

has mandated 16 billion gallons (~60 billion L) of "cellulosic ethanol" be included into the renewable fuel supply (Tao and Aden 2009). Currently the cost of processing is relatively large for cellulosic ethanol, about 70% of the cost of making ethanol, with 30% the raw material cost (Dale 2008). The ultimate goal is to produce cellulosic ethanol at \$0.60-\$0.70/gal (\$0.158 – 0.185/L) making 'cellulosic ethanol competitive with oil at about \$25-30 per barrel' (Dale 2008).

2.1.4 The Limitations of Present (1st Generation) Technologies

First (1st) generation technologies, which form the backbone of the bioethanol industry, have numerous drawbacks in regard to the cultivated biomass presently employed as feedstock. The myriad of issues related to land use patterns and diversion of food supply to bioenergy crops is likely to render 1st generation technologies untenable for long term application. Production of biofuels takes land away from its two other primary uses – food production and environmental preservation (Rajagopal et al. 2007) and even if 100% of arable land was dedicated to bioenergy crops enough biofuels to sate global demand would still not be produced.

Many researchers have cited the benefits of biofuels in terms of their capacity to reduce GHG emissions. Plant biomass offers an attractive alternative (to petrochemical fuels), bypassing the need for fossil resources in chemical production and balancing the time constants of feedstock production and carbon dioxide fixation (van Maris et al. 2006). Biofuels are described as carbon neutral because of their ability to fix carbon dioxide within the same time frame as combustion liberates this gas. The rapid rise in global carbon dioxide levels has come about because of this imbalance between the fixation of CO_2 and the release, the later taking 350 million years and the former taking less than a hundred. However, some argue that biofuels will cause dramatic changes in

land-use patterns which could offset any CO_2 savings derived from the utilization of biomass. Searchinger et al. (2008) contends that land-use changes will cause a net increase in GHG emissions; double GHG emissions over 30 years and cause increases for 167 years. Carbon emissions occur as farmers worldwide respond to higher prices and convert forest and grassland to new cropland to replace the grain (or cropland) diverted to biofuels. There is a cost, in terms of carbon storage and sequestration, to diverting previously unused land or appropriating agricultural land to grow bioenergy crops. Searchinger et al. (2008) suggests the only way to achieve carbon neutrality is to exclusively utilize agricultural wastes.

First generation technologies pose environmental concerns both in regards to possible new GHG emissions related to a change in land use patterns as well as suffering uncertainty in regard to possible GHG savings. Farrell (2006) summarized the findings of six well-to-wheel studies and surmised, 'the impact of a switch from gasoline to ethanol has an ambiguous effect on GHG emissions, with the reported values ranging from a 20% increase to a decrease of 32%.' Most researchers agree that a net decrease in GHG emissions is likely to be observed with the incorporation of ethanol into the fuel supply, most suggest a modest decrease of 13% - 18% (Dale 2008; Farrell 2006; Kim and Dale 2004). Although ethanol, compared to petroleum, releases fewer green house gases upon combustion the magnitude of the total GHG savings must be viewed in light of the primary sources of emission in the ethanol life-cycle, which lie in agricultural practices (34-44%) and petroleum inputs (45-80%) (Farrell 2006).

Diverting food crops for use as substrate in biofuel production has caused unceasing controversy since the inception of the biofuels movement. In 2006, 20% of the US corn crop was diverted to fuel ethanol production. It is estimated that when the fuel ethanol

plants under construction come on-line, increasing the present plant number from 118 to just over 200, more than 50% of the US corn crop will be consumed in the generation of bioenergy. Most authorities agree biofuels have contributed to rising food prices. Much uncertainty exists in this regard and estimates of biofuel contribution range from 15-25% (Sims 2008). The use of corn for ethanol production has increased the prices of US beef, chicken, pork, eggs, breads, cereals, and milk by 10% to 20% (Brown 2008). The chaos of rising prices, for instance, has been seen in Mexico where thousands marched in protest of rising costs of tortilla (Tan et al. 2008). The switch to fuel crops, from other non-energy food crops, could cause additional food security issues. Projected corn ethanol production in 2016 would use 43% of the U.S. corn land harvested for grain in 2004 (Searchinger et al. 2008). According to Searchinger et al. (2008), U.S. agricultural exports will decline sharply, compared to what they would otherwise be at the time, corn by 62%, wheat by 31%, soybeans by 28%, pork by 18%, and chicken by 12%, causing a myriad of problems for importing nations. Such nations will be forced to become increasingly self-reliant, likely appropriating previously unused land for domestic agricultural production. A large scale switch to 'energy plantation' is likely to 'induce structural change in agriculture and change the source, levels and variability of farm incomes' (Rajagopal et al. 2007). In addition, Jacques Diouf, Director General of the UN Food and Agriculture Organization reports that using food grains to produce biofuels already is causing food shortages for the poor of the world (Diouf 2007).

The most salient of counterarguments against 1st generation technologies, despite foment from environmental and food diversion concerns, comes with the reality of their limited supply. Even if you 'dedicated all US soybean and corn production to biofuels you would only meet 12% of gasoline demand and 6% of diesel demand' (Srinivasan 2009).

Globally seven crops (wheat, rice, corn, sorghum, sugarcane, cassava and sugar beet) account for 42% of cropland, if all were dedicated to biofuels this would satisfy just over half of global gasoline consumption. The necessary cultivation of feedstock will always pose limitations to the amount of available biomass for processing. According to Charles et al. (2007) the heightened reliance on 1st generation biofuels may potentially 'inhibit the development and maturation of longer-term alternatives' that could mitigate fossil fuel dependence.

The 1st generation technologies are not a solution to the world's long-term energy needs. Adopting present processing technologies to utilize a feedstock, however, without the necessity of heavy cultivation and diversion of agricultural lands and foodstuffs, could represent a long-term solution to bioenergy generation and sustainable supply. Farrell (2006) claimed 'large-scale use of ethanol for fuel will almost certainly require cellulosic (2nd generation) technology.' First generation technologies offer an imperfect solution whose utility must be viewed as a bridge between total reliance on petroleum and a future energy portfolio where biomass derived alternatives feasibly contribute.

2.1.5 The Transition to 2nd Generation Biofuels

Second (2nd) generation biofuels are made from lignocellulosic biomass feedstock using advanced technological processes (Antizar-Ladislao and Turrion-Gomez 2008). Mature ethanol production relies on minimal pre-processing to liberate free glucose destined for uptake by yeast in conventional fermentation processes. Cellulosic ethanol technologies rely on a distinctly different substrate, that of cellulose; found in leaves, stems, and other structural elements of plants. The conversion of cellulose to ethanol involves the hydrolysis of lignocellulosic biomass to produce reducing sugars. The amount of cellulosic material available for potential use vastly outweighs the amount of

available starch based substrate. A conservative estimate is that presently, there are approximately 400 million tons of biomass available and this number is expected to grow to ~600 million by 2020 (Stephanopoulos 2008). Many agree this could represent a significant contribution to liquid transportation fuel demands when converted to liquid fuels (Rajagopal et al. 2007; Stephanopoulos 2008). Potentials of up to 130-410 EJ/year in 2050, equivalent to 33 to 100% of present energy production, might be available using only abandoned agricultural lands, low-productivity lands and 'rest lands' (Hoogwijk et al. 2005).

The cost of preprocessing cellulosic material to generate free glucose, however, is much higher than that for conventional feedstock, as both mechanical and thermochemical treatments are often required. Cellulosic ethanol technologies, therefore, are still in their infancy, as the cost of processing has been historically prohibitive. Fig. 2.1 illustrates the additional delignification step required to separate the cellulose from the hemicellulosic material, often an expensive and time consuming process (Moxley et al. 2008).

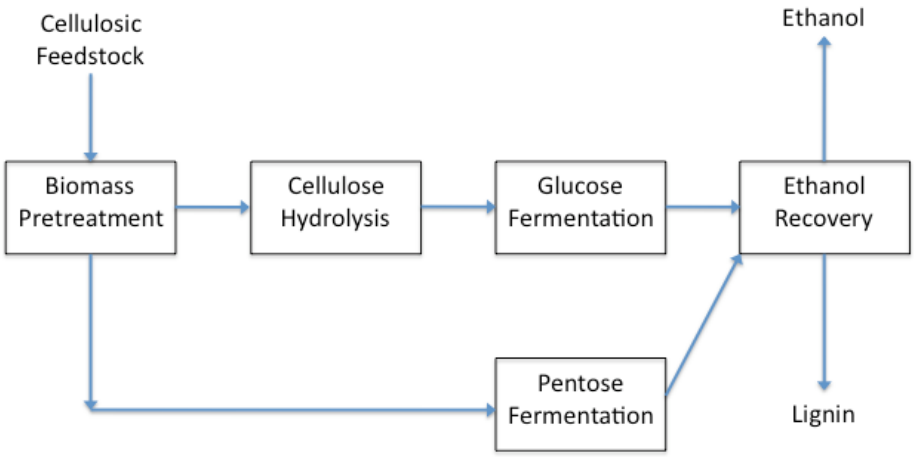

Figure 2.1. Cellulosic ethanol production process. Adapted from IEA (2009).

2.2 Feedstock for Bioethanol Production

2.2.1 Comparison of Conventional Starch Rich Feedstock

In North America the predominant ethanol feedstock is cereal grain based. The fuel ethanol plants in the United States operate almost exclusively on corn feedstock. In 2004 13% of the U.S. corn crop was diverted to produce ethanol (Patzek 2004). In 2009/2010 the USDA projects 4.2 billion bushels of corn will be used to produce ethanol, an increase from 3.6 billion bushels in 2008/2009 (USDA 2009). Overall, ethanol is forecast to command 33% of the corn crop, compared to 30% in 2008/2009. Corn is not (consistently) available in western Canada, resulting in the use of either wheat or barley (Sosulski and Sosulski 1994). In 2009 Canadian wheat and corn based ethanol production reached 487 and 897 million L, respectively (CRFA 2009). Western Canada produced 20

million tonnes of non-durum wheat in 2008 (CWB 2009) and roughly 6.6% of this crop was diverted to fuel ethanol production.[1]

It appears that western Canada's abundance of wheat and relative lack of corn is the major driving factor for the present flourish of wheat-based production. Although wheat has less favorable economics compared to corn, as discussed previously, the production of wheat requires less intensive agricultural practice making it a potentially more sustainable bioethanol substrate. All of the U.S. corn fields are fertilized, with corn consuming an estimated 40% of all nitrogen based fertilizers (Fink et al. 1999), more nitrogen fertilizer than any other crop grown (NAS 2003). Corn erodes soil much faster than natural processes can rejuvenate it, has significant phosphorus requirements (USDA 2007), and uses more insecticides (McLaughlin and Walsh 1998) and herbicides (Patzek 2004) than any other crop grown. Wheat, conversely, requires half the per acre amount of fertilizer and significantly less irrigation and pesticide load.

Grain preprocessing techniques, genetic modification and optimized agricultural practice could address many of wheat's drawbacks by enhancing starch concentration, making wheat comparable to corn. Sosulski (1997) suggests that preprocessing of grain by abrasion can reduce fiber and increase starch concentration, 'increasing plant throughput by 8-23%.' Greater yields (kg/ha) and higher starch content kernels are strategies being explored to improve the economics of wheat based ethanol technologies. These objectives can be accomplished through implementation of new wheat cultivars, application of biotechnology and genetic engineering and use of increasingly sophisticated crop control systems (Koutinas et al. 2004). Agricultural practice in growing

[1] Husky energy, a 260 million L per year ethanol producer in Western Canada, reports a conversion efficiency of 371.4 L ethanol/ tonne grain.

wheat optimized for bioethanol production, especially in regard to nitrogen loading, is an area of active research. Genotype has much less of an effect on grain protein concentration than does applied nitrogen (Kindred et al. 2008) and several reports show that increased nitrogen availability increases protein content in the grain (Kindred et al. 2008; Souza et al. 2004). Protein content is negatively correlated with alcohol yield (Kindred et al. 2008; Swanston et al. 2007). The potential to decrease nitrogen demand of bioethanol tailored wheat has both environmental and economic significance.

2.2.2 Wheat Characteristics Optimal For Bioethanol Feedstock End-Use

The ethanol industry in western Canada is rapidly developing and the need for a sustainable supply of wheat cultivars tailored to meet the demands of that industry is of growing import. The ethanol industry prefers not to scavenge food quality grain from production destined for human consumption (high protein, low starch), and would prefer access to ethanol quality cultivars (high starch, low protein) to ensure access to stable production (Pozniak, 2006). If wheat-based supply chains for bioethanol are to be economically sustainable, the wheat used should have an inherently high potential alcohol yield (Kindred et al. 2008). Wheat cultivars with larger seed size, containing proportionally greater amounts of starch, will ensure that growers can produce grain profitably and provide a consistent supply of good quality feedstock for the ethanol industry (Sosulski and Sosulski 1994). The relationship between large seed size and proportionally greater starch content has not yet been observed in wheat, however.

Ethanol yield, perhaps the most important fermentation performance criteria for the fuel ethanol industry, has been shown to be a starch related property of wheat (Kindred et al. 2008; Lacerenza et al. 2008; Zhao et al. 2009). Obviously a wheat cultivar

with higher starch content in its grain is desirable because it will provide more ethanol per ton of grain and produce smaller amounts of DDGS, resulting in less residual material left over and a greater energy saving during DDGS drying (Zhao et al. 2009). Starch content and turbidity (a measure of the ease of starch release) were shown to be significantly influenced by variety (Kindred et al. 2008; Swanston et al. 2007). Swanston et al. (2007) observed a very strong negative association between alcohol yield and grain protein (nitrogen) content. Higher yielding grains appear to be preferential not only for the proportionally greater amounts of starch but also because larger seed size has a diluting effect on nitrogen content. Consequently, wheat with high yield, high starch/low protein, appear to offer the greatest promise for fuel ethanol feedstock.

The available literature suggests that kernel texture (hard or soft) and growth habit (spring or summer) play a role both in yield and starch content. Wheat is classified into hard, soft and durum categories (Hruskova and Svec 2009), which describe the physical hardness of the endosperm and resistance to reduction to small particles. Durum categories of wheat are not under consideration for fuel ethanol production as both low starch content and high endosperm hardness make them unsuitable. Many studies have reported the increased starch content and ease of handling of soft versus hard wheat. Swanston et al. (2007) states 'distillers experienced some processing problems when hard wheat's were used commercially.'

Grain hardness, however, has not been shown to influence alcohol yield (Swanston et al. 2007) but distillers tend to prefer soft wheat varieties to avoid processing problems (Swanston et al. 2005). Zhao et al. (2009), in a study of thirty U.S. wheat cultivars, found soft nonwaxy endosperm wheat to contain proportionally greater quantities of starch than hard nonwaxy endosperm wheat (Table 2.2). Kindred et al.

(2008), in a study exploring the effects of fertilizer nitrogen on alcohol yield, grain yield, starch and protein content of winter wheat grown in the UK, demonstrated that soft endosperm winter wheat produced 2.9 g/100 g higher starch and 7.7L/tonne more ethanol than hard endosperm winter wheat. In a similar study, conducted on seven spring wheat genotypes, Hard Red Spring (HRS) wheat was shown to have on average 14.5% protein compared to 11.6-11.9% in Soft White Spring (SWS) wheat, suggesting proportionally greater amounts of starch in the soft endosperm variety (Souza et al. 2004). SWS, which contains high levels of starch, gave higher concentrations of ethanol after fermentation than was obtained with high-protein HRS wheat (Sosulski and Sosulski 1994). Breeding programs for soft wheat have likely driven starch content upwards, compared to hard wheat, in accord with their traditional end-use as flours for cakes, pastries and flat-bread. Soft wheat has been targeted as an ideal substrate for bioethanol production as it exhibits both high starch content, soft endosperm texture and has higher yields than hard wheat (Zhao et al. 2009). Soft wheat has also been indicated as possessing lower ratios of amylose to amylopectin (Singh et al. 2009), a physicochemical parameter suggesting high suitability to fermentation feedstock.

Table 2.2. Relative starch content in nonwaxy hard versus nonwaxy soft wheat types. Adapted from Zhao et al. (2009).

Wheat Type		Avg. Starch Content (%, db)
Hard	Red	60.2
	White	63.4
Soft	Red	65.2
	White	66.5

Wheat has two distinct growing seasons. Winter wheat, which normally accounts for 70 to 80 percent of U.S. production but considerably less in Canada due to the harsher winters, is sown in the fall and harvested in the spring or summer. Spring wheat, accounting for 75% of Canadian production, is planted in the spring and harvested in late summer or early fall. Limited research is available on starch content and ethanol yield in spring versus winter wheat. However, several studies suggest winter wheat may have higher yield, starch content and more favorable endosperm texture. In a study of more than 2000 samples of U.S. HRW and HRS wheat, conducted over 3 years, it was found that, on average, HRS had higher protein and hardness than HRW wheat (Slaughter et al. 1992). Protein contents were observed to be 12.7% and 15.4% for HRW and HRS, respectively. Although starch content was not directly measured the well documented inverse relationship between starch and protein content suggests starch content was likely higher in HRW. Seed Manitoba (2007) reported variety trials across the province of Manitoba for 2005/2006 with spring wheat yield ranging from 3042 – 4346 kg/ha (average 3622 kg/ha) and winter wheat yields ranging from 4477 – 5502 kg/ha (average 5394 kg/ha). Not only was winter wheat higher yielding but the reported protein content was also lower, ranging from 10.7 – 12.4% compared to 13.8 – 15.2% for spring wheat.

2.2.3 Western Canadian Wheat for Potential Use as Bioethanol Feedstock

Each milling class of wheat has its own quality and processing characteristics, with varieties from within each class sharing similar physical attributes (CGC 2009b). Six of the eight western Canada classes are of interest to bioethanol producers: Canada Prairie Spring Red (CPSR), Canada Prairie Spring White (CPSW), Canada Western Hard White Spring (CWHWS), Canada Western Hard Red Spring (CWHRS), Canada Western Hard Red Winter (CWHRW) and Canada Western Soft White Spring (CWSWS). The

designation of 'hard' is dropped from CWHRS and CWHRW by the Canadian Wheat board and are preferential described as CWRS and CWRW, respectively. Table 2.3 describes key physical parameters and grain yield for the six wheat classes of interest.

Table 2.3. Kernel size, kernel texture, protein content and average grain yield for 6 classes of western Canadian wheat under consideration for use as fuel ethanol feedstock.

Class	Kernel Size[1]	Kernel Texture[2]	Protein Content[3]	Yield (kg/ha)[4]
CPSR	midsize to large	medium hard	11.50%	3970
CPSW	midsize to large	medium soft	comparable to CPSR	4202
CWHWS	small to midsize	very hard, hard	13.60%	3513
CWHRS	small to midsize	hard	14.1% (13.2% ± 1.4%*)	3653
CWHRW	small to midsize	medium hard	10.8% (10.9 ± 1.2%*)	5198
CWSWS	small to midsize	soft	11.50%	4890**

1 Canadian Grain Commission (CGC) (2009) - Canadian Wheat
2. CIGI (1993)
3. CGC (2007) - Mean Protein for Surveyed Wheat
* CGC (2009) - Western Canada, All Grades Protein Average
4. Seed Manitoba (2007) – All Listed Variety Average
** AC Andrew putative yield is calculated as 135% (Secan 2006) of 2007 AC Barrie yield, as reported in Seed Manitoba 2007.

CPSR/W: These varieties are 1.5-2% lower in protein content than CWHRS varieties, making them lower in price per metric ton (MT) than CWHRS (CGC 2009a). Slightly higher yields for CPSR compared to CWHRS are observed. Annual production of CPSR/W is approximately 1.5 million MT and under 0.5 million MT (and in decline), respectively. CPSW is similar in protein content and yield to CPSR, but it's color was bred to find acceptance in the high quality Asian noodle markets and Middle East flat bread markets, where white wheat is preferred (CGC 2009a). Local availability and high

starch content suggest high-yielding Canada Prairie Spring (CPS) make ideal bioethanol feedstock (Sosulski et al. 1997).

CWHWS: These varieties were bred for use in the Asian noodle market with it's improved yellow alkaline noodle appearance (CGC 2009a). They are considered a minor wheat due to low annual average production. Only two cultivars are registered, AC Kanata and AC Snowbird, both possessing high protein content and similar grain yields to CWHRS.

CWHRS: This is the largest class of wheat grown in western Canada with an annual average production of roughly 15 million MT (CGC 2009a). This wheat possesses a small to midsize kernel with hard texture, high protein content and a low average grain yield of roughly 3650 kg/ha. This grain is exported to over 60 markets annually and is indicated in the use of numerous breads and noodles.

CWRW: This wheat was formerly grown in southern Alberta where winters are milder than the rest of western Canada, but recent production has moved into Manitoba and Saskatchewan with the development of varieties with improved winter hardiness (CGC 2009a) and better disease resistance. Low protein content (2% lower than CWHRS) and very high grain yields (42% higher than for CWHRS) are observed for this class.

CWSWS: This is a low protein, soft wheat, with a high grain yield. In recent years production of this class has been less than 10,000 MT (CGC 2009a). A Western Canadian ethanol feedstock trial, conducted in 2008, demonstrated that SWS wheat produced greater ethanol yields than triticale, CPS or CWRS varieties (Phelps et al. 2009). AC Andrew, the most commonly grown SWS, has generated attention of late as potential

bioethanol feedstock due to its high yield and desirable starch content. For example, Terra Grain Fuels (TGF), a 150 million liters per year (MMly) ethanol producer in Saskatchewan, has targeted AC Andrew as preferred feedstock and is offering local growers monetary incentive to enter into multi-year production contracts for the grain (TGF 2010).

CEGP: Several eastern feed varieties that produce high yields are being considered for registration in western Canada under the Canada Western General Purpose (CWGP) class. According to the Canadian Grain Commission, this class will have no quality requirements and is intended to meet the needs of the feed and industrial sector.

2.2.4 Alternative Cereal Grains as Potential For Bioethanol Feedstock

Canada has several alternative cereal grains that could be used as fuel ethanol feedstock. The replacement of wheat by less expensive feedstock such as rye and triticale would provide good economic opportunities and alternatives for the fuel alcohol industry. Triticale is a cereal produced by crossing wheat (*Triticum*) with rye (*Secale*). Cereal grains such as triticale, rye, and barley, are traditionally lower priced than wheat with all but the latter having similar starch contents (Sosulski et al. 1997). Barley, due to the presence of the hull, has substantially lower starch content on a per seed basis. Studies done on barley show, however, that on a starch basis it is comparable to wheat in terms of ethanol yields and fermentation efficiencies (Sosulski et al. 1997). Rye and triticale are generally 2-5% lower in starch than wheat, but their prices are significantly lower, making the economics favorable.

2.3 Industrial Fermentation Using *Sacchormyces Cerevisiae*

Fermentation wort is an extremely complex environment, consisting of simple sugars, dextrins, amino acids, peptides, proteins, vitamins, ions, nucleic acids and numerous other constituents, and serves as near complete media for *Saccharomyces cerevisiae*, the yeast traditionally used in industrial ethanol production. Yeast are able to use different sugars in the wort as sources of carbon and energy (Montesinos and Navarro 2000b), Pre-fermentation processes ensure favorable pH, assimilable nitrogen content and temperature ranges are achieved for fermentation to proceed smoothly (Panchal and Stewart 1979). However, the yeast lack amylolytic activity and are unable to utilize starch during growth (Birol et al. 1998). The fermentation of cereal grain starch by the ethanol-fermenting microorganism *S. cerevisiae* requires the addition of exogenous endo- and exo-amylases (α-amylase and amyloglucosidase (AMG), respectively), before anaerobic fermentation can occur (Ochoa et al. 2007). Starch based ethanol production utilize bacterial and fungal amylases to cleave starch molecules, constituted by large chain oligosaccharides, into shorter chain dextrans, in a process known as liquefaction. Saccharification, which follows liquefaction, cleaves the pool of short chain dextrans generated by α-amylase into smaller, fermentable sugars, for ultimate conversion to ethanol. The two preparatory steps, where raw cereal starch is converted to fermentable sugars prior to fermentation, referred to as liquefaction and saccharification, respectively, are therefore requirements previous to conducting fermentation.

2.3.1 Starch Based Bioethanol Production

Mature wheat based ethanol technologies typically employ dry milling to grind the wheat mechanically down to a specific particle diameter (Dale and Tyners 2006). Raw starch is insoluble in cold water and in this state is only slowly digested by enzymes

(Holm et al. 1988). Cooking the starch increases enzymatic susceptibility by causing 'rupture and disintegration of the compact crystalline granular structure' (Holm et al. 1988) through a process known as gelatinization. During gelatinization, the starch granules absorb water, swell, exude part of the amylose, become more susceptible to enzyme degradation and lose birefringence (Rooney and Pflugfelder 1986). Wheat starch has gelatinization temperatures in the range of 52 to 54^{o}C (Agu et al. 2006). Industrial processes generally involve the gelatinization of starch-containing raw material with steam, and subsequent liquefaction with α-amylase to dissolve and dextrinize starch carbohydrates; this treatment is referred to as cooking (Das Neves et al. 2006).

Alpha-amylase is classified as an α-(1,4)-glucan glycanohydrolase: attacking at internal glycosidic bond linkages and producing a rapid drop in viscosity (Park and Rollings 1994). The enzyme responsible for liquefaction in most industrial settings is thermostable α-amylase which attacks the α-1,4 glycosidic bonds of starch polymers at random points along the polymer chain (Das Neves et al. 2006). Due to high viscosities and mass transfer problems industrial liquefaction is carried out at the highest temperature possible. The α-amylase derived from *Bacillus licheniformis* is typically added as 0.06% w/w of starch volume and is optimized for temperature at 92^{o}C, it has therefore become the α-amylase of choice for most industrial applications.

The action of α-amylase alone does not produce appreciable amounts of glucose, and that which is produced is a very minor product formed from a very slow secondary reaction of α-amylase on maltotriose and maltotetrose. Instead glucose is produced from the action of AMG on α-amylase degradation products. According to Ao et al. (2007) AMG not only consecutively hydrolyzes α-1-4 linkages, but also hydrolyzes α-1-6

linkages to produce D-glucose from the non-reducing ends of starch and glucose. The hydrolysis of α-1-6 linkages takes place at a much slower rate than that of α-1-4 linkages. The rate of hydrolysis of the α-1-4 linkages of maltose is 28 fold the rate of hydrolysis of the α-1-6 linkages of isomaltose.

The liquefied mash, also referred to as crude mash or wort, is simultaneously saccharified and fermented in one vessel. Immediately after liquefaction, the enzyme AMG is added to the slurry, concomitantly with yeasts, and simultaneous saccharification and fermentation (SSF) is conducted in a single reactor (Das Neves et al. 2006). The action of AMG, an exo-amylase, cleaves the α-1,4 glycosidic bonds at the non-reducing ends of the oligosaccharides in the liquefied wort. The process of saccharification generates only glucose monomers, whereas liquefaction generates an array of oligosaccharides dominated by maltose, maltotriose and larger polysaccharides. Fermentation is performed using *S. cerevisiae* and is carried out at 30-32^{o}C with the addition of ammonium sulfate or urea as nitrogen sources (Sanchez and Cardona 2008).

The process of SSF is preferable to separate hydrolysis and fermentation (SHF) schemes, where starch is hydrolyzed completely into glucose prior to yeast fermentation. SSF has been reported in literature to achieve 'higher rates, yields and concentration of ethanol' (Das Neves et al. 2006) than SHF schemes. SSF schemes appear to optimize ethanol production velocity because it holds glucose concentration at a low level, in fact, as glucose is produced by AMG it is simultaneously consumed by the yeast, resulting in glucose concentrations near zero (Montesinos and Navarro 2000a). Also reported are reductions in fermentation time, risk of microbial contamination and by-product formation. Montesinos and Navarro (2000a) reported a 50% reduction in time between

SHF and SSF schemes, 60 hrs compared to 31 hrs, respectively, to achieve maximum yields. The lower sugar concentrations, and presence of ethanol, retard growth of other competing microorganisms, funneling the majority of the carbon source to the ethanol producing yeast (Montesinos and Navarro 2000a; Wang et al. 2007). Lastly, as reported by Zhao et al. (2009) because AMG and yeast are added simultaneously, a concentrated glucose solution is avoided, and the initial osmotic stress of yeast is lowered. Osmotic stress can cause increased glycerol production, thought to act as an osmoprotectant, and decreases ethanol yields.

The fermented mash is separated into liquid and solid fractions. The stillage consists of the residual, unfermented solids that are dried to produce a high protein animal feed known as Dried Distillers Grains with Solubles (DDGS). The ethanol fraction is typically concentrated using conventional distillation and then passed through a molecular sieve to dehydrate the mixture. The product mixture has to be dehydrated in order to be used as a fuel oxygenate, the form in which ethanol is employed in the transportation sector (Cardona and Sanchez 2007). Yields of ethanol as high as 90 to 95% of the theoretical maximum have been realized under industrial conditions (Thomas and Ingledew 1990).

2.3.2 The Role of Fermentable Sugars in SSF

In most facultative anaerobes, organisms capable of existing in both oxygen rich and oxygen poor environments, oxygen depletion controls the switch from respiration to fermentation. In *S. cerevisiae* this switch is controlled not only by oxygen depletion but also in response to external glucose levels (Otterstedt et al. 2004). Yeast will utilize fermentation as the essential energy source in the presence of sufficient concentration of fermentable sugar, even if oxygen is available. Aerobic ethanol production by *S.*

cerevisiae is thought to depend on the relative capacities of the fermentative and respiratory pathways: high glucose levels result in a glycolytic rate exceeding that of the pyruvate dehydrogenase (Pdh) reaction, thereby generating an overflow towards pyruvate decarboxylase (Pdc) and hence ethanol production (Otterstedt et al. 2004). *S. cerevisiae* does not produce ethanol in the presence of oxygen and low external glucose concentrations, but rather depends entirely on respiration under these conditions (Kappeli 1986). Industrial ethanol producers generally do not maintain strict anaerobic conditions but maintain high sugar concentrations, enabling the yeast to remain essentially fermentative throughout the run.

Yeast consume different sugars as sources of carbon and energy. When raw wheat starch is used as substrate for fermentation, the liquefied wort has a typical sugar composition: maltose (27%), maltotriose (4%), glucose (1%) and non fermentable glucose polymers (68%) (Montesinos and Navarro 2000a). The great majority of yeast process strains consume both maltose and maltotriose only after glucose depletion (Salema-Oom *et al*. 2005). Glucose is the preferred carbon source for *S. cerevisiae* and complex regulatory control circuits have evolved to ensure that the expression of alternative sugar utilization enzymes is repressed when glucose is present in the growth medium (Needleman 1991). The repressive effect of glucose on the metabolism of alternative carbon sources is considered to limit the productivities of industrial fermentations (Lee et al. 1995). According to Montesinos and Navarro (2000a) after glucose repression has been lifted the yeast will consume maltose and maltotriose, however, the trisaccharide is very often incompletely consumed. Maltotriose has the lowest priority for uptake by the yeast and displays slower uptake rates than for maltose (Zastrow et al. 2000). A common problem encountered by a number of breweries is the incomplete utilization of wort

maltotriose (Zheng et al. 1994). Since maltotriose is the second most abundant fermentable sugar in the brewery wort, its incomplete fermentation results in a loss of fermentation material (Panchal and Stewart 1979) and lower ethanol yields. Although maltotriose is considered a fermentable sugar, Zastrow et al. (2001) observed that maltotriose is mainly respired by industrial strains and that the rate-limiting step for fermentation was active transport across the plasma membrane, which is dependant on the expression of a single low affinity transport protein.

Maltose, the dominant sugar in the wort, is the most significant sugar in the process regulation of SSF because it is substrate not only for AMG but also for the yeast (Montesinos and Navarro 2000a). At the beginning of the SSF process maltose is the major sugar in the wort but will not normally be consumed until 50% of the initial glucose concentration has been exhausted (Ernandes et al. 1993), as a consequence of the previous noted glucose repression effect. Maltose has a repressing effect on AMG, reducing its activity by twenty fold. Montesino and Navarro (2000b) suggest once maltose consumption begins in earnest by the yeast, AMG inhibition imparted by the maltose appears to lift, resulting in an increase in glucose production rate. Once maltose repression on AMG is lifted, AMG acts to generate glucose from any of the oligosaccharides present, preferentially attacking the longer glucose polymers (Montesinos and Navarro 2000b). Glucose production from the AMG does not appear to create a second repression effect on maltose uptake because as the glucose is generated via AMG it is consumed by the yeast, resulting in low levels of glucose concentration in the media for the remainder of the fermentation period.

2.3.3 Yeast Fermentation Thermodynamics

S. cerevisiae when grown under anaerobic conditions converts glucose to pyruvic acid via the glycolytic pathway and ultimately converts pyruvic acid to ethanol and CO_2 via fermentation (Bai et al. 2008). Ethanol, a primary metabolite, is tightly coupled with cell growth. The two ATPs produced in glycolysis (Reaction 1.1) are used to drive the biosynthesis of yeast cell growth. Without the continuous consumption of ATP by the growing yeast the increased ATP concentration signals the cessation of glycolysis, halting ATP production and further growth (Bai et al. 2008). Reaction 1.2 depicts the chemical conversion of glucose to ATP under aerobic combustion, known as respiration. Respiration is a far more efficient energy generator than fermentation but cannot proceed without oxygen.

Reaction 1.1. Alcoholic Fermentation

$$\underset{\text{(Glucose)}}{C_6H_{12}O_6} \rightarrow \underset{\text{(Ethanol)}}{2\ C_2H_5OH} + \underset{\text{(Carbon Dioxide)}}{2\ CO_2} + \underset{\text{(Adenosine Triphosphate)}}{2\ ATP} + \text{heat}$$

Reaction 1.2. Respiration (complete oxidation)

$$\underset{\text{(Glucose)}}{C_6H_{12}O_6} + \underset{\text{(Oxygen)}}{6\ O_2} \rightarrow \underset{\text{(Carbon Dioxide)}}{6\ CO_2} + \underset{\text{(Water)}}{6\ H_2O} + \underset{\text{(Adenosine Triphosphate)}}{36\ ATP} + \text{heat}$$

The calculation for theoretical ethanol yield, given in Appendix A, from a known concentration of glucose assumes 100% conversion efficiency. In reality there is a small percentage of the glucose that is lost as energy to the cell and is not converted to metabolic end-products. Conversion efficiency to produce ethanol by fermentation is usually 90-95% (Wu et al. 2006). Theoretical ethanol yields for ethanol production are, therefore, not reported as the calculated maximum of 51.1 % ethanol (w/w) but rather

closer to 47% ethanol (w/w). The reason for the imperfect conversion efficiency may include incomplete hydrolyses of starches, glucose consumption funneled to fueling cell growth, or the inevitable production of by-products during ethanol fermentation (Wu et al. 2006).

2.3.4 Yeast Fermentation Primary By-product Formation

Glycerol, produced at a level of about 1.0% (w/v) for most ethanol fermentations, is the main metabolic by-product (Bai et al. 2008). Glycerol is produced in yeast to help maintain intracellular redox balance and as a response to osmotic stress. Higher mash pH, increased osmotic pressure, lower flux of pyruvate due to the use of glycolytic intermediates subsequent to the step in the pathway producing reduced NAD for biosynthesis all can stimulate the conversion of dihyroxyacetone phosphate (D-HAP) to glycerol (Ingledew 1999). Glycerol production lowers ethanol yield and is therefore unfavorable (Wang et al. 2007).

Acetic acid is a by-product of ethanol production and inhibits fermentation in an exponential way (Krisch and Szajani 1997). The mechanism of its toxicity involves the acidification of the cytoplasm and modifying certain enzymes of glycolysis (Pampulha and Loureirodias 1990). Acetic acid is thought to be formed from the oxidation of acetaldehyde (Eglinton et al. 2002).

Other by-products such as organic acids and higher alcohols are produced at much lower levels (than glycerol) (Bai et al. 2008). Industrially, both lactic acid and acetic acid generation are monitored as concentrations of lactic acid between 0.2-0.8% (w/v) are known to stress S. *cerevisiae*. Yeast generate small quantities of lactic acid through its metabolic flux. However, contaminating bacteria such as Lactobacilli, which convert glucose to lactic acid, are also known to cause lactic acid build up. In general, low lactic

acid concentrations are used to indicate the lack of infection during fermentation (Wang et al. 2007), as lactic acid produced via metabolism is considered negligible.

2.4 The Effect of Physicochemical Properties of Wheat Starch on Enzymatic Hydrolysis and Fermentative Alcohol Yield

Starch is the predominant component of wheat grain, constituting 60- 65% of the kernel on a dry weight basis. The functional properties of starch vary widely across botanical origin (Swinkels 1985) and between cultivars of a species. Wheat cultivars with different characteristics are desirable for different final product application (Franco et al. 2002). Gluten quality and starch characteristics, for example, play important roles in the textural quality of bread. Wheat starches destined for use in noodle making are scrutinized for characteristics that affect eating quality (Hung et al. 2006; Zhao et al. 2009). Starch composition such as amylose content, branch chain-length distribution of amylopectin (Jane et al. 1999), lipid content, starch granule size distribution (Raeker et al. 1998; Wilson et al. 2006) and granular architecture (Zhang and Oates 1999) have all been shown to affect the functional properties of starch. Lacererenza et al. (2008) recently pointed out that the traditional selection for milling and baking quality is not consistent with maximal ethanol yield per hectare. The lack of breeding programs for wheat varieties designed specifically for ethanol production, suggests Swanston et al. (2007), is due to a lack of appropriate selection procedures due to limited understanding of the factors contributing to alcohol yield.

Of particular interest to the bioethanol industry are the functional properties of starch lending themselves to ease of amylolytic hydrolysis and high conversion efficiencies of starch to fermentable sugars. The bioavailability of starch may differ among grain cultivars and may affect the conversion rate and final yield of ethanol

(Moorthy 2002). In general, as compared to non-cereal starches, wheat and other cereal grains have been observed to have low resistance to enzymatic degradation (Zhang and Oates 1999). Amylose/amylopectin content (Lee et al. 2001; Wu et al. 2006; Zhao et al. 2009), granule size and distribution (Liu et al. 2007), extent of molecular association between starch components (Dreher et al. 1984), degree of crystallinity (Ao et al. 2007; Zhang et al. 2008b), and amylose-lipid complexes was (Guraya et al. 1997; Holm et al. 1988; Kwasniewska-Karolak et al. 2008) have all been reported in literature to have bearing on gelatinized starches susceptibility towards α-amylase.

Recent research into optimized bioethanol production has focused on the development of new and improved cereal and maize hybrids with higher starch contents to increase ethanol yields (Wu et al. 2006). Grain preprocessing strategies are also being investigated and have the potential to 'increase throughput rate and capacity of ethanol plants' (Sosulski and Sosulski 1994). The processing of higher starch feed streams, achieved through adoption of new process technology or higher starch grains, is the inevitable outcome of present biofuel development. Starch content, although inarguably the most critical feature in determining ethanol conversion efficiency, is not the only influential parameter in understanding fermentation performance.

Starch with a high intrinsic resistance to enzymatic hydrolysis, although of possible high compositional content in the kernel, could have reduced availability for conversion to fermentable sugars and would perhaps make an erroneous selection as bioethanol feedstock. A more thorough understanding of the influence of starch structural and physicochemical properties on the efficiency of liquefaction, saccharification and fermentation is therefore indicated.

Research focused on the relationship between the physicochemical properties of starch and conversion to fermentable sugars, addressing the unique goals of the bioethanol industry for optimized feedstock, has not been sufficiently addressed in the literature. The review that follows draws upon research conducted in nutritional science, food bioprocessing, carbohydrate chemistry, and the limited studies available within the field of biofuel crop development to explore the role of starch structure and function to its potential for amylolytic degradation and, when available, fermentation performance.

2.4.1 Amylose & Amylopectin

Starch granules are composed of two types of alpha-glucans, amylose (Fig. 2.2) and amylopectin, which represent approximately 98-99% of the dry weight (Tester et al. 2004b). The ratio of the two polysaccharides varies according to the botanical origin of the starch but within cereal grains maintains ranges of 25-28% amylose and 72-75% amylopectin (Hung et al. 2006). Starches with less than 5% amylose are considered waxy wheat, where starches with greater than 35% amylose are considered high-amylose starches (Wu et al. 2006). Amylose is an essentially linear molecule, consisting of α-(1,4)-linked D-glucopyranosol units with a degree of polymerization (DP) in the range of 50-6000 glucose residues. It is now well recognized that a fraction of the amylose molecules is slightly branched by α-(1,6)-linkages. In contrast, amylopectin is a very large, highly branched chain molecule with a DP ranging from 30,000 to 300,000 glucose units and

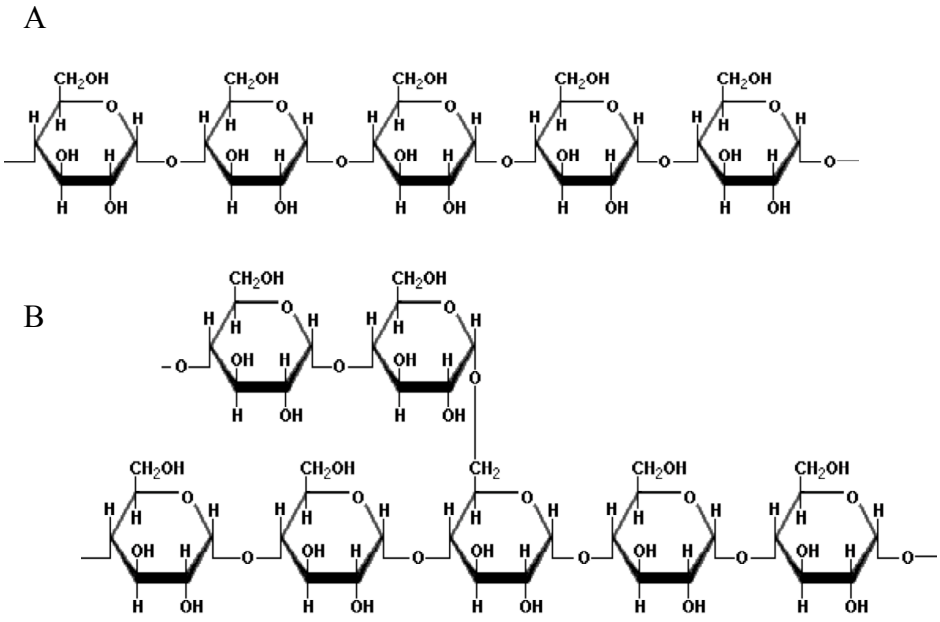

Figure 2.2. Amylose molecules composed of glucose monomers connected as in (A) via α-(1,4)-glycosidic bonds or as in (B) with infrequent branch chains via α-(1,6)-glycosidic bonds.

consists of α-(1,6)-linked D-glucopyranosol units attached to α-(1,4)-bonds (Zobel et al. 1988). A strand of amylose and amylopectin are respectively reported to have molecular weights in the range of 10^4-10^6 and 10^7-10^8 daltons, respectively.

Wu et al. (2006) studied high-amylose starches and demonstrated that amylose content, more than protein or fiber content, had significant effect on ethanol fermentation efficiency. The study revealed that conversion efficiency decreased as amylose content increased. Starch in its native form is resistant to enzymatic digestion and must be gelatinized, changing it into an amorphous mass, before it becomes susceptible to glucosidic enzymes. Because of the existence of starch granules with high gelatinizing temperatures, and the formation of amylose-lipid complexes (AML) and reassociation of amylose molecules during gelatinization and enzymatic hydrolysis, there is always some starch that escapes hydrolysis by amylolytic enzymes (Boltz and Thompson 1999). As much as 2% of the starch in industrial dextrose production remained undigested as insoluble particles in the hydrolysate (Hebeda and Leach 1974). Amylose is likely to form amylose-lipid complexes in the caryopsis or during mashing that are resistant to enzymatic hydrolysis (Wu et al. 2007). Wu et al. (2006) states that, in most instances, resistant starch content increases as the amylose content in starch increases.

High amylose containing starches also inhibit fermentative ethanol production by yielding high viscosity mashes. Greater enzyme dosing, reported by Wu et al. (2006), wasn't an effective strategy for increasing maltose and dextrin formation. It appears that high mash viscosity creates a bottleneck to enzymatic activity that can only be overcome with greater reaction time; greater reaction time, however, provides increased opportunity for deleterious retrogradation and amylose-lipid complexing, increasing the degree of insoluble sugars.

A reduction in amylose content has been positively correlated with enzymatic digestibility and represents the current accepted paradigm for the pattern of α-amylolysis on native starches. Fermentation studies consistently report higher ethanol yields on waxy (high amylopectin) substrates than non-waxy counterparts. A high percentage of amylopectin seems to be more susceptible to α-amylase during fermentation, 'with higher gas production of waxy wheat flour as compared with the nonwaxy wheat flour' (Lee et al. 2001). Wu et al. (2007) reported waxy and hetero-waxy sorghum hybrids to generally have higher conversion efficiencies than nonwaxy hybrids. Zhao et al. (2009) studied the effects of waxy vs. nonwaxy soft and hard wheat's for fuel ethanol production and found results consistent with Lee at al. (2001) and Wu et al. (2007). High ethanol conversion efficiency of waxy wheat were reported as compared to nonwaxy wheat, 95.4 – 96.2% and 92.6%, respectively (Zhao et al. 2009). Wu et al. (2006) states that 'conversion efficiencies increased as the amylose content decreased, especially when the amylose content was >35%.' Employing waxy wheat as feedstock for fuel ethanol production has been recommended (Lacerenza et al. 2008). However, waxy wheat has not yet reached the commercial stage of development and little is known about industrial fermentation performance.

2.4.2 Starch Granule Morphology

One feature of the endosperm of mature Triticum species is the multimodal starch granule size population (Wilson et al. 2006). Present research suggests that wheat starch has a trimodal distribution (Raeker et al. 1998; Wilson et al. 2006). The large disc-shaped A granules appear 4 days after anthesis and continue to increase in size throughout the grain-filling period (Ao and Jane 2007). The intermediate-sized granules (B-type) and the smallest granules (C-type) are thought to be initiated at specific times after anthesis,

depending on cultivar, growing location, and isolation method (Wilson et al. 2006). A-type granules make up the bulk of the starch, but are fewer in number than the smaller sized B- and C-type granules. A-type granules have been reported as 10-35mm in average spherical diameter and account for more than 70% of the total starch mass, but less than 10% of the granules by number. Small granules account for over 90% of the granules by number, but less than 30% of the total starch by weight in the endosperm (Lindeboom et al. 2004). The granule types have been shown to differ in their structure. Amylose content was highest in large wheat starch granules, while lipid content was highest in small granules (Morrison and Gadan 1987). The two types of granules (A- and B-) differ in their ratio of amylopectin to amylose, differ in size and quantity (Ao and Jane 2007) and have differing ratios of amylose to bound lipids (Ao and Jane 2007; Raeker et al. 1998). Additionally, the amylopectin of large granules has been shown to contain a greater number of long amylopectin 'B' chains and had lower fractions of certain side chain lengths, than did small granules from the same cultivar (Lindeboom et al. 2004). Raeker et al. (1998) suggests, however, that variation within a cultivar, especially for starch granule size distribution and phospholipid content, are significantly influenced by environmental factors indicating that predictive traits for a given cultivar may be difficult to assess.

Starch composition, gelatinization and pasting properties, enzyme susceptibility, crystallinity, swelling and solubility are all affected by granule size (Lindeboom et al. 2004). In a study performed by Liu et al. (2007) the in vitro digestibility of A- and B-Type granules from soft and hard wheat flours was investigated. The digestion of gelatinized starch, measured at 20 minutes and 2 hours, with pancreatic α-amylase and

AMG showed higher resistant starch content in the hard A-type wheat granule as compared to the B-type granule. The results were similar for soft wheat showing slightly higher resistant starch content in the soft A-type granule as compared to the B-type. Liu's findings suggest that B-type wheat granules contain less resistant starch as compared to A-type granules, and that soft wheat may contain less resistant starch than hard wheat.

In cereal starches there are small quantities of naturally occurring lipids which are capable of forming complexes with amylose (Kwasniewska-Karolak et al. 2008). The presence of amylose-lipid complexes (AMLs) negatively influence production of glucose syrups because it reduces water binding and swelling of starch granules, thus impairing the access of amylolytic enzymes (Matser and Steeneken 1998). Several studies have reported higher amylose content in the A-type granule, explaining, in part, their increased resistance to enzymatic hydrolysis. Absolute amylose content of wheat and triticale for the A-type granule were measured as 30.9% and 28.2% and that of the B-granule were 25.5% and 19.7% (Ao and Jane 2007). Liu et al. (2007) reported apparent amylose content as 25.4 and 25.8% for A-type (soft and hard wheat, respectively) and 16.5 and 19.3% for B-type.

The selection of wheat cultivars with a high proportion of the B-type granule polymorph may yield benefit to starch conversion efficiencies. The quantity of B- versus A-type granules appears to vary significantly across cultivars. However, a comparison of reported values across cultivars is challenging given that 'genotype, environment, and method of analysis' (Stoddard 1999) all appear to be influential in assessing the proportions of starch type in the granule. Various studies have reported B-granule distributions in the range of 25-40% (Dengate and Meredith 1984) to 13-34% (Soulaka

and Morrison 1985) for wheat starch. Recent studies have yielded more detailed information regarding variation between wheat cultivars. For example, hard red winter (HRW) wheat was found to have 48.4% by volume classified as B-type (<10 μm diameter) (Bechtel et al. 1990). In another study B-granules occupied volumes in the range 28.5 – 49.1% (mean 39.9%) for HRW wheat, while hard red spring (HRS) wheat B-granules occupied volumes in the range 37.1 – 56.2% (mean 47.3%) (Park et al. 2009). In a study by Raeker et al. (1998) thirty-four starch samples from soft wheat cultivars were investigated for differences in particle size distribution. It was found that contributions from the large granule population (>9.9 μm) to the total volume were 57.9 – 76.9%; B-type particles (<9.9 μm) therefore represent a range of 23.1 - 42.1% of total volume.

The literature available, although suffering discrepancy in sampling and analytical method, suggests that a high fraction of B-granules may lead to an optimized substrate for liquefaction. In particular, the study of Liu et al. (2007) suggests that soft wheat with a high abundance of B-type granules might be best suited for fermentation feedstock.

2.4.3 Amylopectin Fine Structure

Starch is stored as discrete semicrystalline granules in higher plants (Gupta et al. 2009). The granule crystallinity is associated with the amylopectin component (Jenkins and Donald 1995). According to Jenkins and Donald (1995) the currently accepted amylopectin structure involves short amylopectin chains forming double helices and associating into clusters. These clusters pack together to produce a structure of alternating crystalline (double helices) and amorphous lamellar composition (amylopectin branch points). These semi-crystalline growth rings, consisting of alternating crystalline and amorphous lamellar, are bound by amorphous growth rings, the alternation of which make up the granule architecture. The branched chains of amylopectin, according to the cluster

model of amylopectin proposed by Hizukuri et al. (1986), can be fractionated into B_3, B_2, B_1 and A-chains, as depicted in Fig. 2.4.

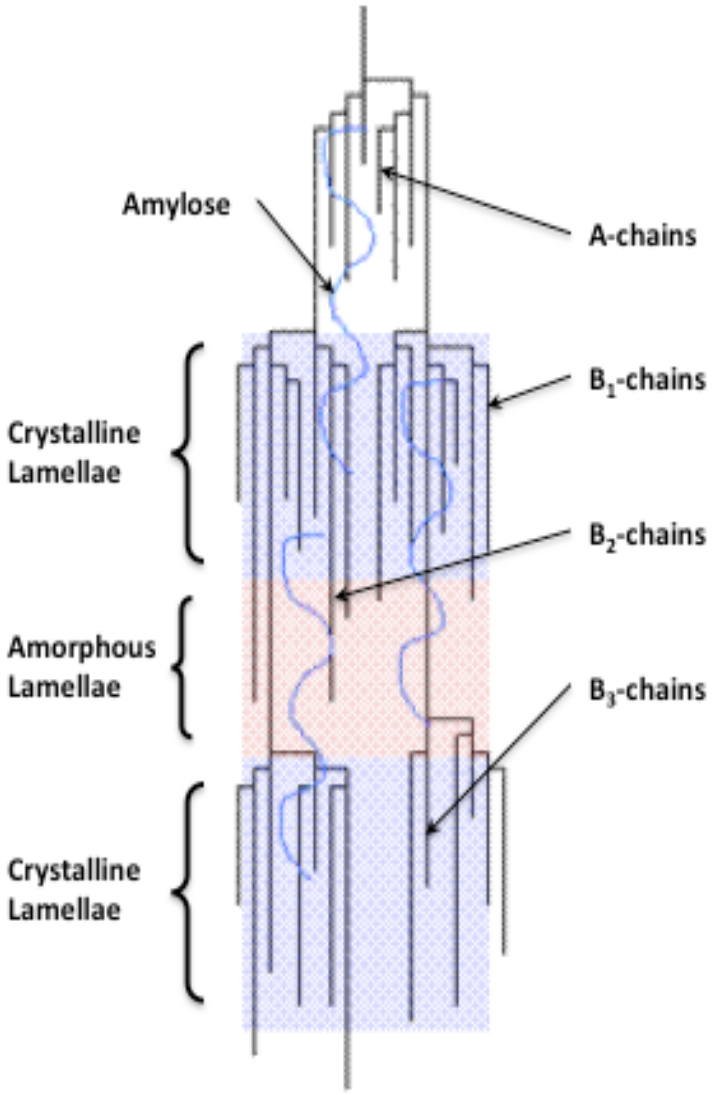

Figure 2.4. Structure of amylopectin depicted as 'cluster model' proposed by Hizukuri (1986). A and B_1 chains dominate the distribution and occur as parallel strands, believed to wind into left-handed double helices. Larger B chains are thought to transverse two, three and four clusters. Short A-chains posses dp 6-12, B_1 chains range from dp 13-24, next B_2 chains occur at dp 25-36, and finally B_3 and longer chains at dp>36. Adapted from Hizukuri (1986).

The dominance of certain fractions of side chain length dictates the type of crystallinity displayed during x-ray diffraction studies, referred to as A-type, B-type and C-type (Jenkins and Donald 1995). Most cereal starches possess A-type crystallinity and have higher weights and number percents of short A chains (Chung et al. 2008). Amylose is thought to exist mainly in the non-crystalline state (Hizukuri et al. 1996) but the exact location of amylose within the granule interior and the extent of its interaction with amylopectin is unclear (Gupta et al. 2009). It is likely that a large portion is found within the amorphous growth ring, with only small amounts associated with the semi-crystalline growth ring (Jenkins and Donald 1995).

During starch gelatinization, starch granular or supramolecular structure is disrupted, resulting in the pattern of enzymatic hydrolysis being 'predominently related to the inherent molecular structure of amylopectin' (Zhang et al. 2008b). The relationship between the molecular structure of starch (amylopectin fine structure) and its digestion rate after starch gelatinization is not well understood (Zhang et al. 2008a). The two key features of amylopectin fine structure are chain length distribution and branching pattern. Little variation in the branching pattern of amylopectin has been reported in normal wheat starches. Hanashiro (1996) reported the following distribution of amylopectin side chains after debranching: DP 6-12, 27%; DP 13-24, 49%; DP 25-36, 14%; and DP >37, 10%. These findings were supported in a study of 126 starch samples selected from the *Triticum-Aegilops* group, of which 16 samples were derived from Triticum *aestivum*, or common bread wheat (Yasui et al. 2005). Additionally, in a study of 192 club and soft white winter wheat, no detectable difference in amylopectin structure was found (Lin and Czuchajowska 1997).

Several studies have reported the effects of increasing branch density of amylopectin on digestion rate of gelatinized starches through partial shortening of amylopectin exterior chains (Ao et al. 2007; Zhang et al. 2008a; Zhang et al. 2008b). Ao (2008) reports that starch products exhibiting high branch densities, with shorter average chain lengths, showed reductions in rapidly digested starch of up to 30% and concomitant increases in slowly digested starch of up to 20%. Zhang (2008) found that amylopectin of maize starch with either a high or low ratio of short chain fraction (SF, DP < 13) to long chain fraction (LF, DP > 13) showed increased quantities of slowly digested starch. The amylopectin fractions in this study exhibiting the lowest quantities of slowly digested starch had SF/LF ratios of 0.5, exhibited in wild type maize. The inherent molecular structure of amylopectin with a higher amount of branches and shorter chains (high SF/LF ratio) is not favorable for rapid enzyme digestion (Zhang et al. 2008b). Average chain length for soft wheat starches has been reported as DP 25.6-26.9 (Franco et al. 2002). Future work is needed to quantify the average amylopectin chain length optimal for rapid enzymatic digestion during liquefaction.

2.4.4 Thermal Properties

Gelatinization, as previously described, precedes liquefaction in the fermentation process and describes the physical break down of granular starch into solubilized, amorphous polymers readily hydrolyzed by α-amylase and AMG. This irreversible loss of native structure occurs when sufficient energy is applied to break intermolecular hydrogen bonds in the crystalline areas (Rooney and Pflugfelder 1986). Gelatinization temperature is considered a parameter of crystallite perfection (Tester and Morrison 1990). Amylopectin is primarily responsible for starch crystallinity and the presence of amylose lowers the melting temperature by decreasing crystallinity (Gupta et al. 2009). Van Hung

et al. (2006) states 'waxy wheat starch with predominant amylopectin requires higher energy for gelatinization caused by its higher crystallinity as compared to non-waxy and high-amylose wheat starches.' Two endothermic peaks are seen when thermal properties are determined using differential scanning calorimetry (DSC). The first peak represents the starch gelatinization and the second peak corresponds to the melting of amylose-lipid complexes (Hung et al. 2006). Gelatinization temperatures and enthalpies associated with gelatinization endotherms vary between starches. In a study by Gupta et al. (2009) native wheat and corn starch, measured at 70% moisture content, were reported to have onset temperatures (To) of 60.19 and 70.12^{o}C, respectively, peak temperature (Tp) of 64.06 and 73.85^{o}C, and conclusion temperature (Tc) of 68.42 and 78.20^{o}C.

Limited research has been performed to elucidate the relationship between gelatinization temperature and industrial fermentation efficiency. From an energy standpoint low gelatinization temperature starch may be favorable as feedstock to produce fermentation-based products due to lower temperatures required to efficiently process the grain. Low gelatinization temperatures, however, are associated with high amylose content starch (Hung et al. 2006; Noda et al. 2002). Although amylopectin rich starch has a higher gelatinization temperature evidence suggests that it dissociates more completely. Zhao et al. (2009) found waxy wheat starch to have complete disruption/dissolution of the granule at 70-80^{o}C, compared to non-waxy cultivars which showed evidence of intact granular structure under hot-stage microscopic visualization for temperatures as high as 90^{o}C. Wu et al. (2007) reports that waxy starches easily gelatinize and have concomitantly high conversion efficiency. Wu et al. (2007) claims that cooking high-amylose starches at higher temperatures, with shearing, may significantly increase the digestibility of the high-amylose starches and therefore improve conversion efficiency.

However, even at temperatures above 120^{o}C the conversion efficiency achieved for high amylose starches was still significantly below that of normal or low amylose starches. Given the myriad of conversion inefficiencies related to the enzymatic hydrolysis of high amylose starch their adoption as ethanol feedstock is unlikely.

The relationship between amylopectin fine structure and the thermal properties of starch have been well established (Franco et al. 2002). Starch that consists of amylopectin with a larger proportion of long branch-chains displayed higher gelatinization temperatures and enthalpy changes (Franco et al. 2002; Jane et al. 1999). Several studies have reported the manipulation of branch chain length to modify this physical parameter. Amylopectins with more long branch chains produce more ordered double-helical crystallites, which require higher temperatures to uncoil and dissociate (Franco et al. 2002; Huang et al. 2007; Song and Jane 2000). Conversely, higher contents of extremely short chains within the amylopectin, DP 6 and 7, appear to lower T_0 (temperature of onset of gelatinization), T_p (gelatinization peak temperature) and DH (gelatinization enthalpy) (Noda et al. 2002). In regards to bioethanol production, driving down the gelatinization temperature by manipulating amylopectin branch chain length may lead to a substrate with lower energy requirements to achieve high conversion efficiency.

2.4.5 Pasting Properties

Pasting viscosity profiles are analyzed using a Rapid ViscoAnalyzer (RVA), a typical profile is observed in Fig. 2.5. Pasting temperature is the point when the temperature rises above the gelatinization temperature, inducing starch granule swelling and viscosity increases. The peak viscosity indicates the maximum viscosity reached during the heating and holding cycle and is indicative of the water holding capacity of starch (Gupta et al. 2009), peak temperature occurs at peak viscosity. The breakdown

viscosity is normally regarded as a measure of the disintegration of the starch granules as they are heated (Agu et al. 2006) due to granules rupturing and soluble amylose leaching out. The degree of RVA breakdown is related to the solubility of the starch, and the more soluble the starch, the more it will thin on shearing (Hoseney 1998). As the mixture is cooled, re-association between starch molecules, especially amylose, results in the formation of a gel and subsequent viscosity increases. Total setback involves retrogradation, or re-ordering, of the starch molecule.

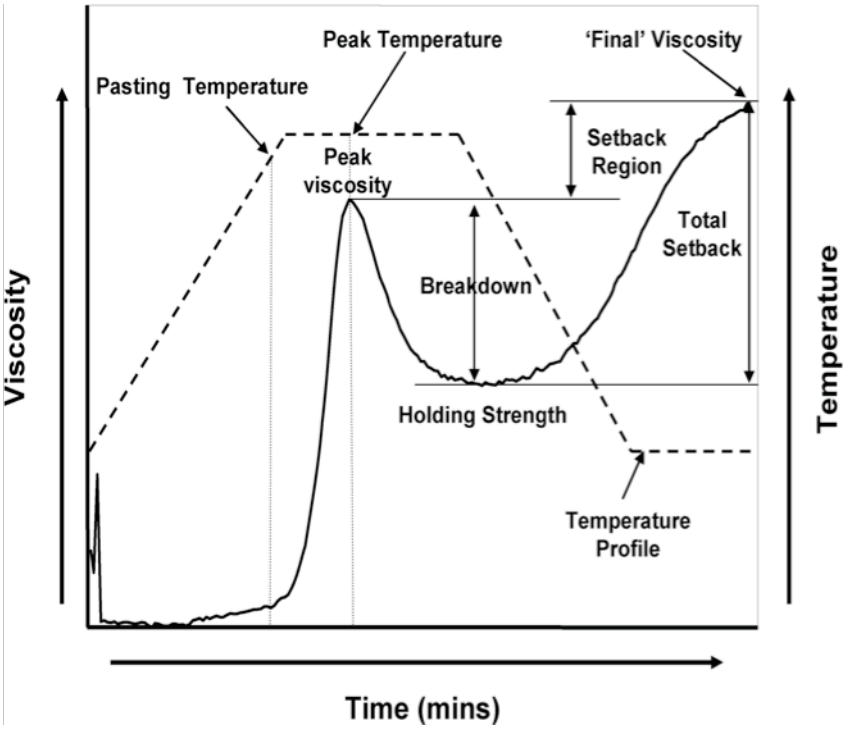

Figure 2.5. A typical RVA pasting profile showing the commonly measured parameters.

Pasting properties of starch are affected by amylose and lipid contents and by branch chain-length distribution of amylopectin (Gupta et al. 2009). Starches with larger amylose, lipid and phospholipid content have higher pasting temperatures, lower peak viscosity and shear-thinning (breakdown viscosity), and higher setback viscosity (Jane et al. 1999; Zeng et al. 1997). Waxy wheat flour, conversely, has been shown to have

significantly lower peak and pasting temperature, higher peak viscosity and lower setback viscosity than non-waxy or normal wheat flour (Abdel-Aal et al. 2002; Gupta et al. 2009; Zeng et al. 1997). Waxy starch swells rapidly and swollen granules degrade at lower temperature, indicating that waxy starch rapidly develops viscosity but cannot maintain the stability of paste viscosity (Gupta et al. 2009). Zhao et al. (2009) examined waxy starch granules using hot-stage microscopy and demonstrated that these starches rupture more extensively, even without mechanical shearing, and disperse more readily than nonwaxy counterparts. Waxy wheat starch has been described as having lower final viscosity (Abdel-Aal et al. 2002; Graybosch 1998; Zeng et al. 1997) an indication that it has less ability to retrograde and form strong gels.

Pasting properties, like thermal properties, are 'affected by the branch chain length distribution of amylopectin' (Jane et al. 1999). According to Franco (2002) amylopectin with longer branch chains display larger peak viscosity and lower pasting temperatures than shorter chain counterparts. Jane et al. (1999) also reported that long chains with DP>50 accelerated retrogradation of amylopectin, whereas the short chains (DP 6~9) retarded it. It is plausible that very long chains of amylopectin mimic amylose to form helical complexes with lipids and intertwine with other branch chains to hold the integrity of starch granules during heating and shearing (Gupta et al. 2009).

Wu et al. (2007) described ideal feedstock for ethanol production as having rapid liquefaction characteristics and low viscosity during liquefaction. A high viscosity in the mash may impair the accessibility of starch to the enzyme and, thus, delay the liquefaction process (Wu et al. 2007). The ideal pasting properties, as depicted by RVA analysis, of a starch destined for use as bioethanol substrate include high solubility, demonstrated by a low viscosity after breakdown, and limited ability to retrograde upon cooling,

demonstrated by a low final viscosity. Modified RVA analysis was performed by Zhao et al. (2009) to assess the viscosity of waxy versus non-waxy wheat during gelatinization and liquefaction. In this case, conventional RVA analysis was modified to include dosing with α-amylase, providing a metric for the balance between gelatinization and liquefaction. Zhao et al. (2009) states that 'for waxy wheat cultivars, gelatinized starch granules were more susceptible to breakdown under liquefaction conditions; thus, starch molecules were more extensively exposed and more accessible to heat-stable α-amylase, so lower peak viscosities were obtained. Due to the low peak viscosity for waxy wheat during liquefaction, the dry-grind industry could thus increase the solids content in the mash, lower α-amylase dosages, or decrease energy requirements for stirring systems when waxy wheat is used as a feedstock.'

2.5 Selection Criteria for Wheat as Bioethanol Feedstock

Elite genotypes for ethanol production have been described as having rapid liquefaction characteristics, low viscosity during liquefaction, high fermentation speed and high fermentation efficiencies (Wu et al. 2007). Identification of genetic factors within wheat cultivars contributing to these parameters is a topic that has received little attention to date. Wheat cultivars producing 'feed class' grain with high starch content, and thus relatively low protein content, have been highlighted as the preferred ideotype for ethanol production (Kindred et al. 2008; Sosulski and Sosulski 1994). High starch, low protein content and high yield are reported as the most critical features of high ethanol producing wheat varieties. However, critical to starch conversion efficiency to fermentable sugars is the solubility of starch during gelatinization and the availability of solubilized material to liquefying enzymes. Therefore, a salient feature in the generation of cultivars tailored to the needs of the bioethanol industry include physicochemical

parameters of starch lending themselves to high conversion efficiency under the conditions of liquefaction. High conversion efficiencies of starch to fermentable sugars will result in the greatest quantity of ethanol produced per unit of raw substrate when displayed in grains that exhibit both high yield and high starch content.

Starch properties conferring high conversion efficiencies to fermentable sugars, based on the available literature, are indicated in Table 2.4. Of particular note is the relationship of amylopectin to the majority of indicated parameters, and the marked benefit to each, in regards to bioethanol application, of an increase in amylopectin and concomitant decrease in amylose content. The encompassing recommendation of the present review is the selection of wheat with the highest amylopectin content achievable, theoretically delivering starch optimized for both rapid and complete degradation by industrial enzymes.

Table 2.4. Ideal Physicochemical Parameters of Starch Recommended for Use as Bioethanol Feedstock.

Parameter	Ideal Condition for Optimized Bioethanol Production	Reference
Amylopectin/Amylose Content	>75% Amylopectin	Wu et al. 2006 Wu et al. 2007 Zhao et al. 2009
Starch Granule Morphology	High ratio of B-type granule	Liu et al. 2007
Amylopectin Fine Structure	High proportion of long to short chain fraction	Ao et al. 2007 Zhang et al. 2008a/b
Thermal Properties	High solubility	Wu et al. 2007 Zhao et al. 2009
Pasting properties	Low breakdown, final & liquefaction viscosity	Zhao et al. 2009

Chapter 3: Materials & Methods

3.1 Whole Grain Chemical Composition and Physicochemical Analysis of Starch

3.1.1 Materials

No. 1 or 2 grade whole wheat and triticale kernels from 15 cultivars; S700PR, AC Crystal, AC Vista, HY 475, AC Barrie, Superb, Snowbird, SWS 162-008, Bhishaj, AC Andrew, CDC Falcon, Buteo, Hoffmann, Nass and Banjo (triticale), grown at select locations in western Canada during the 2007 crop were used in this study. Quality characteristics of whole kernel samples were measured to establish grade. Quality characteristics of interest included test weight per bushel and weight per 1000 kernels; performed according to AACC approved method 55-10.01 and CGC (2010) weight per 1000 kernels test, respectively. Most samples were grown at Melita, Manitoba, and provided by Dr. Anita Brule-Babel, Department of Plant Science, University of Manitoba. Additionally, Dr. Brule Babel provided breeder seed of the triticale, Banjo. AC Andrew, Snowbird, Falcon and Buteo were sourced separately and provided by Brad Pickering of Secan (Kanata, Ontario, Canada). AC Andrew was grown in Carmongay, Alberta. One sample of whole kernel Central Manitoban corn, provided by Husky Energy's Minnedosa Ethanol Facility, was used in this study as a baseline for wheat performance comparison.

3.1.2 Sample Preparation and Whole Grain Analyses

A cleaner-separator (Labofix, Emceka, Germany) removed the impurities from the grain seeds subsequent to milling (Udy, Fort Collins, CO) through a 0.5mm screen. Moisture content in the grain was determined after heating at 130^{o}C for 1 h in a semi-automatic moisture oven (CW Brabender, South Hackensack, NJ, USA). Protein content (%N x5.7) was determined by combustion nitrogen analysis (model FP-248 Leco Dumas CNA analyzer, St Joseph, MI, USA) calibrated with ethylenediamine tetra-acetic acid

according to the AACC International Approved Method 46-30. Total starch content was determined enzymatically using Megazyme kit (Megazyme International Ireland Ltd., Co. Wicklow, Ireland) according to the AACC International Approved Method 76-13. The protocol was modified such that after 0.2mL of aqueous ethanol (80% v/v) was added to wet the sample, 2.0mL of DMSO was added, vortexed, and placed in a boiling water bath for 5 min. Total arabinoxylan (AX) content was determined colorimetrically by the phloroglucinol method of Douglas (1981). Ash content was determined according to the AACC international Approved Method 08-03.

3.1.3 Starch Isolation

Whole kernel samples were milled for 25-30s using a blender to achieve a coarse flour. To a sealable flask 100mL of 0.1M HCl was added to 10g of ground whole kernel sample. The flask were then placed on its side in a shaker, at medium speed, set at 4^0C and left overnight. The samples were warmed to room temperature and 0.2M NaOH was added to achieve a neutral pH of 5.5. Samples were centrifuged at 4000g for 15 min (Beckman Avanti^{TM} Centrifuge J-25I with FIBERLite^{R} F10BCI-6X500y rotor, Beckman Coulter, Inc., Brea, CA., USA). The liquid layer was removed by aspiration and discarded. The grist was scraped into a mortar, and 8ml of 0.1M Tris/HCl buffer containing 0.5% sodium metabisulfite at pH 7.0 was added to each bottle to rinse it. The sample was ground gently in a mortar until a paste had formed. The grist and liquid were returned to the centrifuge bottle and the mortar was quantitatively rinsed back into the centrifuge bottle with 22.5mL total volume of buffer. In a separate container, 100μL B-xylanase (8000 U/ml) (Megazyme International Ireland Ltd., Co. Wicklow, Ireland) and 835μL Proteinase K (600 U/ml) (F.Hoffmann-La Roche Ltd., CH-4070 Basel, Switzerland) was added to 12.5mL of Tris buffer and mixed. To each centrifuge bottle,

2.5mL of this enzyme mixture was added, for a total volume of 25mL. Bottles were sealed, and placed in a shaking water bath at 30 ^{0}C overnight. Digested grist was sieved in a 75μm Allen Bradley sieve, until no chunks of starch remained, and the liquid collected. Liquid containing starch was transferred to screw cap 50mL centrifuge tubes and spun at 28000g in a centrifuge for 10 min (Beckman Avanti^{TM} Centrifuge J-25I with FIBERLite^{R} JA-17 rotor, Beckman Coulter, Inc., Brea, CA., USA), supernatant was discarded and the sample resuspended in a small amount of water and spun again. The pellet was resuspended in 4mL of water. Into 20mL of 80% CsCl, placed in a centrifuge tube, the sample was then carefully layered on top of the CsCl solution. The sample tube was rinsed with 2mL of water, and this was bubbled under the sample layer using a Pasteur pipette and air pump in the centrifuge tubes. Tubes were centrifuged at 28000g for 30 min. The protein layer and remaining CsCl solution layer were removed with a Pasteur Pipette and discarded. The starch was washed with water, shaken, and centrifuged at 28,000g for 15 min. The supernatant was removed and the starch was washed 5 more times. Finally, the starch was suspended in about 3ml water and filtered through a 0.45μm PALL GH Polypro hydrophilic polyprene membrane filter (PALL Corporation, Mississauga, ON). A small amount of acetone was used to remove the residual moisture and the dry pellet was transferred to a fume hood for approximately 2 day before grinding in a mortar and pestle.

3.1.4 Amylose/Amylopectin Ratio

Amylose content of starch was analyzed following the method of Gibson et al. (1997) using an amylose-amylopectin assay kit (Megazyme International Ireland, Co. Wicklow, Ireland). The following modifications to assay protocol were employed. 25mg of wheat sample was weighed into a 10mL Kimax tube, instead of 20-25mg of sample.

Starch Pretreatment was employed with both purified and whole grain samples of wheat. During starch pre-treatment the samples were dissolved in 1mL DMSO with vigorous mixing while slowing heating to boil. Samples were vortexed continually until boiling achieved. The samples were then left to boil for 15 min, with intermittent vortexing. The samples were then mixed with 6mL of ethanol and placed on ice for 30 min until a white precipitate was formed. The tubes were then centrifuged at 4000g for 10 min. The supernatant was discarded and the tubes inverted and left to drain for 5 min.

3.1.5 Branch Chain-Length Distribution of Amylopectin

The extracted wheat starches from each sample were gelatinized and then purified with 90% dimethyl sulfoxide, and ethanol precipitation as described by Jane and Chen (1992). The dried purified starch (25mg) was redissolved in 1N NaOH, diluted with water and neutralized with 1N HCl. The starch solution was autoclaved for 5 min (121ºC). The dissolved starched was subsequently incubated with isoamylase (Hayashibara Biochemical Laboratories Inc., Okayama, Japan; 500 U/g, 0.1M sodium acetate buffer, pH 3.5) for 17 hours at 40ºC. After digestion, samples were placed in a boiling water bath for 15 min to inactivate the enzyme and then filtered through a 0.45μm GHP Acrodisc syringe filter prior to injection (PALL Corporation, Mississauga, ON). The amylopectin branch chain-length distributions were analyzed by high performance anion exchange chromatography (HPAEC) using a Waters 625LC pump, 717plus WISP autosampler (Waters Associates, Milford, MA), a Dionex CarboPac PA1 column (4 x 250mm) with a PA1guard column and a Coulochem III (ESA, Chelmsford, MA) electrochemical detector equipped with a 5040 Analytical cell containing a gold target electrode. The Coulochem III detector pulse parameters were E1 = +200mV, E2 -1000mV, E3 = +600mV, E4 = -100mV; T1 = 500ms, Acquisition Delay (AD) = 300ms,

T2 = 10ms, T3 = 1ms, T4 = 10ms, current range = 2uC; recorder out at +1 V; and a baseline offset of 0%. The eluents A and B were 150mM NaOH and 150mM NaOH containing 500mM sodium acetate, respectively. The gradient of eluent B was 25% at 0 min, 50% at 15 min, 75% at 45 min, 90% at 60 min, and 100% at 72 min. The mobile phase was held at 100% B for 0.5 min before returning to starting conditions (75% A and 25% B). The column was then allowed to equilibrate for 20 min giving a total run time of 92.5 min at a flow rate of 1 mL/min. The data was processed using Waters Empower Software by defining a baseline for each sample and calculating each peak area as a percentage of the total area from the detector response to the baseline.

3.1.6 Particle Size Distribution

Malvern Mastersizer 2000 (Malvern Instruments, Southborough, MA) was used to measure particle size distribution. Sample concentrations were within equipment recommendations and the refractive indicies of 1.31 for water and 1.52 for starch were used as the standard. The isolated wet starches were slurried with water and vibrated with ultrasound for 3 min on 50% intensity as pre-treatment to ensure particle agglomeration was minimized.

3.1.7 Thermal Properties

Differential scanning calorimetry (DSC) measurement was conducted using a SSC 5200 with a DSC 120U (Seiko Electronics, Tokyo, Japan) calibrated with indium. For gelatinization studies, 15.0mg of bone dry starch was weighed into silver pans with 35µL of distilled water. After sealing, pans were scanned at 1 ^{0}C/min from 40 to 120^{0}C.

3.1.8 Pasting Profiles

A Rapid-Visco Analyser (RVA) (Newport Scientific, Warriewood, Australia) was used to measure the apparent viscosity of samples as a function of temperature, time and

stirring. Suspensions (10% w/w dry mass basis) were freshly prepared of purified starch samples (purification protocol outlined above) and loaded in a Series 3 D RVA utilizing Thermocline for Windows Version 1.1. The starch suspension was stirred rapidly at 960 rev/min for 10s before the shear input was decreased and held constant at 160 rev/min for the heating and cooling cycles. The suspension was heated from 50^0C to 95^0C in 4 min 42s, and held at 95^0C until 7 min 12s, before cooling to 50^0C for 3 min 48s.

3.1.9 Gelatinization, Liquefaction and Reducing Sugar Profiles

Suspension (10% w/v as is) of purified starch was prepared in 0.1M phosphate buffer at pH 6.4. The starch was placed in a 100mL culture flask with a stir bar and heated on a stirring hot plate set to medium heat and the lowest stirring speed for 10 min or until the starch had visually gelatinized. The samples were autoclaved for 15 min. exposure time at 121^{o}C with no exhaust time. A blank and two replicates were prepared for each sample. The samples were immediately removed from the autoclave and placed in an 84^{o}C water bath and allowed to equilibrate for 30 min. The two experimental samples were dosed with 150μL (10 KNU-S/g-starch) of α-amylase (Novozyme's Tetramyl (334.6 KNU-S/mL), Novozymes North America Inc., Franklin, N.C., USA), the blank with additional phosphate buffer. The samples were swirled by hand at 15 min intervals for 90 min. Upon removal from the water bath 10.5mL of 0.1M citric acid was added to each sample, dropping the pH to 2.6 and inactivating the enzyme. The samples were then transferred to 50mL volumetric flasks and the quantity of reducing sugars measured according to the neocuproine test (Dygart, 1965) using the sample blanks as background for spectrophotometric analysis.

3.1.10 Molecular Weight Determination of Soluble Sugars After α-Amylolyis

Weight-average molecular weight (M_w) of the gelatinized starches was carried out with a high performance size exclusion chromatography (HPSEC-MALS) system which consisted of a Waters chromatography system, Alliance 2695, 2487 UV detector controlled by Waters Empower software (Waters Associates, Milford, MA), and a DAWN HELEOS laser-light-scattering detector, Optilab rEX refractive index detector, controlled by Astra software (Wyatt Technology, Santa Barbara, CA). Separation was performed on two columns in series, SB802HQ and SB803MHQ (300 x 7.8mm; Shodex Showa Denko K.K. Tokyo, Japan) with a guard, OHPAKSB-G (50 x 6mm; Shodex) maintained at 30^{o}C. A mixture of sugar and pullulan standards were prepared to cover the range of molecular weight of interest. The samples (50μL inject) were analyzed using an effluent of 0.075M sodium nitrate, 0.02% sodium azide buffer at a flow rate of 0.5 mL/min over 60 min. The gelatinized starch samples were dissolved in milli-Q water and boiled for 10 min, cooled and made up to 5mL in a volumetric flask. A portion of this was used for digestion with porcine pancreatic alpha-amylase (Sigma-Aldrich Canada, Oakville, Ontario) at 20 and 120 min, at 40°C. The samples were boiled to inactivate the enzyme, cooled and make up to 5mL with a volumetric flask. Blanks with and without enzyme were also prepared. All samples were filtered through a 0.45μm GHP filter (PALL Corporation, Mississauga, ON). The standards were used to prepare a column calibration needed for molecular weight determination. Astra uses the equation of log (molar mass) from the conventional calibration and the concentration from the Optilab RI to calculate the weight average molecular weight.

3.2 Fermentation Analysis of Select Starches

3.2.1 Materials

No. 1 or 2 whole wheat and triticale kernels from 6 cultivars; S700PR, HY 475, Snowbird, AC Andrew, CDC Falcon, Buteo, and Banjo (triticale), were selected based on their physicochemical parameters for inclusion in fermentation analysis. Corn from central Manitoba was also included to establish a baseline for ethanol conversion performance.

3.2.2 Starch isolation for fermentation

To a 500mL sealed flask 45g coarsely ground whole grain was added to 400mL of 0.02 M HCl. The flasks were then placed on their side in a shaker, at medium speed, set at 4^0C and left overnight. The samples were warmed to room temperature and 0.2M NaOH was added to achieve a neutral pH of 7.0, before centrifugation at 4000g for 15 min (Beckman Avanti^{TM} Centrifuge J-25I with FIBERLite^{R} F10BCI-6X500y rotor, Beckman Coulter, Inc., Brea, CA., USA). The liquid layer was removed by aspiration and discarded. The grist was scraped into a mortar, 0.1M Tris/HCl buffer containing 0.5% sodium metabisulfite at pH 7.0 was added to each bottle to rinse it. The sample was ground gently in a mortar until a paste formed. The grist and liquid were returned to the centrifuge bottle and the mortar was quantitatively rinsed back into the centrifuge bottle with 125mL total volume of buffer. To each bottle 100μL endo B-xylanase M1 (2000 U/mL) (Megazyme International Ireland Ltd., Co. Wicklow, Ireland) and 1.25mL Proteinase K (600 U/mL) (F.Hoffmann-La Roche Ltd., CH-4070 Basel, Switzerland) was added. Bottles were sealed, and placed in a shaking water bath at 30^0C overnight. Digested grist sieved in a 75μm Allen Bradley sieve and the liquid collected. Any chunks of starch remaining were mashed into the sieve and washed with water until all starch

passed through. The samples were spun for 20 min at 2000g and the resultant supernatant was discarded. The gray material left covering the surface of the starch was scraped off. The starch was then re-suspended in distilled water and spun for 20 min at 2000g. This was repeated two more times, the final re-suspension done in 1% NaCl solution before being spun and rinsed again with distilled water. Using vaccum filtration the starch slurry was then passed through a 15.0cm Whatman #2 filter. The sample was allowed to dry overnight.

3.2.3 Substrate Preparation

Roughly 2.5g of oven dried purified starch was placed in a 225mL baffled bottom Erlenmeyer flask with metal cap and stir bar. Added to the flask was 35mL of 0.1M sodium phosphate buffer at pH 6.0. The starch/buffer mixture was swirled by hand until visibly homogeneous and then heated on a stirring hotplate, with low speed mixing, for ten minutes until gelatinization had occurred. The mixture was then autoclaved at 121^0C for 15 min without a drying cycle, ensuring homogeneity of gelatinization and sterility. The gelatinized mixture was then placed in a shaking water bath at 84^0C for 30 min to equilibrate to temperature, using aseptic technique 75μL (10 KNU-S/g-starch) of α-amylase (335 KNU-S/mL) (Tetramyl, Novozymes North America Inc., Franklin, N.C., USA) was then added. The mixture was left for 90 min in a shaking water bath at 84^0C and swirled by hand at 30 min intervals to ensure no lumps had formed. The gelatinized starch was then removed from the bath and left to cool to room temperature before transfer to a sterile 50mL volumetric flask. Sterile 0.1M sodium phosphate buffer at pH 6.0 was used to quantitatively transfer the gelatinized starch up to volume.

3.2.4 Preparation of Innoculum

Active dried yeast (Bio-Ferm® XR, North American Bioproducts Corporation, Duluth, GA, USA) was cultured in YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) and incubated overnight at 32^0C before streak plating on YPD agar (YPD broth + 20 g/L agar). Plates were incubated at 32^0C for 3 days before being placed in a 4 ^{0}C fridge to stop growth. Isolated colonies were selected and used to inoculate 100mL sterile YPD broth. Cells were incubated overnight at 32 ^{0}C with shaking (200 rpm). The growth of yeast cells was established using optical density by spectrophotometry (BioMate 3 UV-Vis Spectrophotometer, Thermo Scientific, Waltman, MA, USA) at 600 nm (OD_{600}) The yeast cells were harvested if the OD_{600} was between 2-3. The starter culture of yeast was aliquoted to ensure a cell concentration in the 50mL serum bottle achieved an initial OD_{600} of 0.4-0.5, thus ensuring uniform initial cell concentrations between experimental samples. Aliquots of cell culture were portioned out and centrifuged for 5 min at 6000g (Sorvall RC 6 plus with rotor SS-34, Thermo Scientific, Waltmas, MA, USA). The supernatant was discarded and the cells washed with 1mL of sterile dH_20, vortexed, and spun down again. The supernatant was discarded and the cells were re-suspended in 2mL of sterile sodium phosphate buffer at pH 6.0. To the buffer containing the cells 5.3µL (10 AGU/g-starch) of amyloglucosidase (AMG) (945 AGU/mL) (Spirizyme, Novozymes North America Inc., Franklin, N.C. USA) was added and the mixture vortexed.

3.2.5 Experimental Design

Batch experiments were conducted using a Simultaneous Saccharification and Fermentation (SSF) scheme with liquefied starch substrates at concentrations of 10 g/L. Serum bottles (Bellco Glass Co., Vineland, New Jersey, USA) with a working volume of

50mL were used as reactors for all batch tests. Serum bottles were prepared by adding 10 g/L Yeast Extract and 20 g/L Peptone in sodium phosphate buffer at pH 6.0. The serum bottles were air-sealed with butyl rubber stoppers and aluminum seals before being gassed and degassed (1:4 min) four times with 100% nitrogen according to the protocol of Daniels et al. (1986). Serum bottles were then autoclaved to ensure sterility. To the sterile media 10mL of starch substrate and the previously prepared innocculum, containing yeast and saccharifying enzyme, was added. Three replicates and a blank of each sample were prepared, the blank containing only media and starch preparations. In lieu of innoculate, 2mL of buffer containing saccharifying enzyme, at the previously prescribed dosage, was added. At the end of experimental runs select sample blanks were plated on YPD-agar and grown up at 32^0C for 2 days to ensure no contamination was present.

3.2.6 Cell Growth

Growth of *S. cerevisiae* on starch was measured using optical density by spectrophotometery at 600 nm. Samples (2mL) were drawn from each of the experimental and blank vials and OD_{600} was determined using the sample blank as the spectrophotometric zero reference. Due to the action of AMG, each time point required the preparation of a new blank to compensate for the decreasing cloudiness of the starch solution. A growth curve relating OD_{600} to cell forming units/mL (cfus/mL), established from streak plate counts done in triplicate, was used to determine cell density in experimental cultures. After collection and OD_{600} measurement the samples were centrifuged at 6000g for 5 min (Sorvall Legend Micro 21R Centrifuge, Thermo Scientific, Waltmas, MA, USA), the cells discarded and the supernatant collected and stored at -20^0C.

3.2.7 Fermentation End-Product Analysis

All samples were thawed, centrifuged and passed through a 0.45μm filter before analysis. Lactic acid, acetic acid, glycerol and ethanol were simultaneously measured with the profile of fermentable sugar (glucose, maltose, maltotriose and maltotetrose) using High-pressure liquid chromatography (HPLC). The separation of all target compounds was carried out on an Aminex HPX-87H cation exchanger column (7.8mm i.d. x 300mm) (Bio-Rad, Hercules, USA) and an Aminex Micro Guard Cation H precolumn (Bio-Rad, Hercules, USA). A 0.002 N sulfuric acid mobile phase at a flow rate of 0.6 mL/min was employed. The column was maintained at 60^0C. Injection volume of undiluted samples was 5μL for all runs. Target compounds were detected using an Agilent G1362 refractive-index detector (Agilent Technologies Canada, Inc., Missasauga, Ontario, Canada) maintained at 45^0C.

3.3 Statistical Analysis

All statistical analyses were executed using SAS statistical software (release 9.1.3; SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine differences in mean values based on data collected from replications of each measurement. Significance was established at $P \leq 0.05$. All samples were measured in at least triplicate for fermentation studies and at least duplicate for purified starch analysis. RVA analysis, molecular weight determination of soluble sugars, protein and ash content were not measured in duplicate and therefore no statistical analysis was possible. Liquefaction performance analysis by molecular weight determination was also not available in duplicate and therefore no statistical analysis was possible. Linear regression analysis was performed with Excel 2004.

Chapter 4: Whole Kernel Composition and Physicochemical Properties of Select Western Canadian Wheat and Their Starches

4.1 Introduction

Biofuels, such as ethanol and bio-diesel, are poised to serve as not only an agent of greenhouse gas reduction but also as a means to secure an energy supply that is local, renewable and independent of a financially volatile and potentially unreliable oil market. According to the Canadian Renewable Fuels Association (2009) Canada produced approximately 1.39 billion L/year of ethanol as of January 2009 drawing upon wheat, corn, wood waste, municipal landfill waste and lignocellulosic straws from wheat, barley and oats. Canada will need to produce 2 billion L/year of ethanol by 2010 to meet the 5% federal renewable fuel standard recently mandated (CRFA 2009). Wheat (*Triticum aestivum*) is cultivated worldwide primarily as a food commodity (Kindred et al. 2008) but in western Canada the abundance and local availability of this grain make it well poised to serve as the backbone for a growing bioethanol industry. In 2009 Canadian wheat and corn based ethanol production reached 487 and 897 million L, respectively (CRFA 2009). Western Canada produced 20 million tonnes of non-durum wheat in 2008 (CWB 2009) and roughly 6.6% of this crop was diverted to fuel ethanol production.[2]

Starch is the predominant component of wheat grain, constituting 60- 65% of the kernel on a dry weight basis. The functional properties of starch vary widely across botanical origin (Swinkels 1985) and unique characteristics are desirable for different final product application (Franco et al. 2002). Lacererenza et al. (2008) recently pointed out that the traditional selection for milling and baking quality is not consistent with

[2] Husky energy, a 260 million L per year ethanol producer in Western Canada, reports a conversion efficiency of 371.4 L ethanol/ tonne grain.

maximal ethanol yield per hectare. The lack of breeding programs for wheat varieties designed specifically for ethanol production, suggests Swanston et al. (2007), is due to a lack of appropriate selection procedures due to limited understanding of the factors contributing to alcohol yield.

Recent research into optimized bioethanol production has focused on the development of new and improved cereal and maize hybrids with higher starch contents to increase ethanol yields (Wu et al. 2006). Grain preprocessing strategies are also being investigated and have the potential to 'increase throughput rate and capacity of ethanol plants' (Kindred et al. 2008; Sosulski and Sosulski 1994) by decreasing non-starch carry through. The outcome of present biofuel development is the inevitable processing of higher starch feed streams, achieved through adoption of new process technology or higher starch grains. Ethanol yield, perhaps the most important fermentation performance criteria for the fuel ethanol industry, has been shown to be a starch related property of wheat (Kindred et al. 2008; Lacerenza et al. 2008; Zhao et al. 2009). Obviously a wheat cultivar with higher starch content in its grain is desirable because it will provide more ethanol per ton of grain and produce smaller amounts of dried distillers grains (DDGS,) resulting in less residual material left over and a greater energy saving during DDGS drying (Zhao et al. 2009).

Starch content, although inarguably the most critical feature in determining ethanol conversion efficiency, is not necessarily the only influential parameter in understanding fermentation performance. Starch with high intrinsic resistance to enzymatic hydrolysis, although of possible high compositional content, could yield a low sugar load to yeast, making it an erroneous selection as feedstock, especially when considering the industries move towards ever higher starch feed streams. Of particular

interest to the bioethanol industry are the functional properties of starch lending themselves to ease of amylolytic hydrolysis and high conversion efficiency of starch to fermentable sugars. The bioavailability of starch may differ among grain cultivars and may affect the conversion rate and final yield of ethanol (Moorthy, 2002). A more thorough understanding of the influence starch structural and physicochemical properties have on the efficiency of gelatinization and liquefaction, the two most relevant industrial processes in the preparation of bioethanol feedstock, is therefore indicated. The following five parameters have been shown to influence the functional properties of starch and are used as evaluative criteria in this study: amylose/amylopectin content (Lee et al. 2001; Wu et al. 2007; Wu et al. 2006; Zhao et al. 2009); starch granule morphology (Liu et al. 2007); amylopectin fine structure (Ao et al. 2007; Sasaki et al. 2002; Zhang et al. 2008a; Zhang et al. 2008b); thermal properties (Wu et al. 2007; Zhao et al. 2009); and pasting properties (Zhao et al. 2009).

Wheat cultivars producing 'feed class' grain with high starch content, and thus relatively low protein content, have been highlighted as the preferred ideotype for ethanol production (Kindred et al. 2008; Sosulski and Sosulski 1994). High starch and yield characteristics coupled with starch optimized for rapid and complete degradation by industrial enzymes should represent ideal feedstock for the industry. Presently no evaluative criteria of grain starch quality, as it relates to maximized ethanol yield, are available to bioethanol producers. This study attempts to quantify the inherent variability of starch physicochemical properties from western Canadian wheat cultivars with the aim of identifying those starches well suited for bioethanol end-use.

4.2 Materials & Methods

Material and methods for wheat starch analysis is described in sections 3.1.1 to 3.1.9 of this thesis. Statistical analysis was performed according to section 3.3.

4.3 Results & Discussion

4.3.1 Whole Kernel Quality Characteristics

Kernel characteristics for wheat and triticale are displayed in Table 4.1. Test weight, which provides an indication of flour yield and density of the wheat (Maghirang et al. 2006), showed a range amongst wheat samples of 72.4 to 83.2 kg/hL. In a study by Maghirang et al. (2006) using 100 samples of HRW and 98 samples of HRS wheat grown in the U.S, both classes were found to yield test weights of approximately 61 lb/bu (79 kg/hL), which is consistent with the average wheat test weight of 76.7±3.2 kg/hL reported here. The triticale, however, had a test weight of ~70 kg/hL, roughly 7 kg/hL lower than the wheat sample average. Thousand kernel weight (1000KWT) showed considerable variation between wheat samples with a range of 25.2 to 38.3g, the average observed value of 32.9±4.4 g, however, appears to be consistent with previously reported values (Hui et al. 2006). Observed 1000KWT for the triticale sample included in this study was found to be ~46g and appears to be ~13g higher than the average 1000KWT for wheat (~33g).

Several authors have indicated the benefit of increased grain yield to the bioethanol industry. More productive crops, in regards to starch produced per hectare of dedicated land, may drive down feedstock cost or require the dedication of fewer lands to achieve similar production capacity. High test weight and 1000KWT, therefore, represent a potentially ideal condition for bioethanol end-use. Consistent with Kleijer et al. (2007), no statistically significant relationship was observed between 1000KWT and test weight

for wheat included in this study. However, starch content, discussed in the proceeding section, was found to have positive correlation coefficients with 1000KWT and test weight, ($r=0.56$, $P<0.05$ and $r=0.51$, $P<0.05$, respectively), indicating higher density and higher weight seeds may contain greater quantities of starch than lower density/weight counterparts.

Table 4.1. Whole kernel quality characteristics including moisture content, test weight and thousand kernel weight test (1000KWT).

Class[a]	Variety	Moisture (% as is)	Test Weight (kg/hL, dwb)	1000KWT (g, dwb)
CPSR	S700 PR	9.54	78.2	35.59
	AC Crystal	10.68	74.2	30.53
CPSW	AC Vista	10.34	76.0	38.33
	HY 475	8.76	76.1	35.26
CWRS	AC Barrie	9.66	75.8	29.13
	Superb	10.15	73.0	28.31
CWHWS	Snowbird	9.16	81.0	38.04
CWSWS	SWS 162-008	10.22	74.6	33.70
	Bhishaj	9.20	72.4	26.36
	AC Andrew	9.56	80.6	38.62
CWRW	CDC Falcon	9.70	83.2	34.99
	Buteo	9.43	78.8	32.80
CEGP	Hoffmann	10.44	75.2	33.80
	Nass	8.86	74.6	25.20
Triticale	Banjo	8.79	69.9	45.90
CMC	Corn	11.13	—	—

[a] Canada Prairie Spring Red (CPSR), Canada Prairie Spring White (CPSW), Canada Western Red Spring (CWRS), Canada Western Hard White Spring (CWHWS), Canada Western Soft White Spring (CWSWS), Canada Western Red Winter (CWRW), Canada Eastern General Purpose (CEGP), Central Manitoba Corn (CMC)

[*] No replicates were performed for test weight, values for 1000KWT and moisture are averages of n=2

4.3.2 Chemical Composition of Raw Materials and Starches

Composition of the whole grain flour was found to vary significantly among samples for starch and arabinoxylan content (Table 4.2). Statistical analysis was unavailable for protein content but wide variance was observed with average grain protein concentrations ranging from a high of 16.8% for AC Barrie, a Canada Western Red Spring (CWRS), to a low of 9.9% for Central Manitoban Corn (CMC). Average protein content for wheat was observed to be 14.3%, in line with previous findings for spring wheat (Sosulski and Sosulski 1994; Wang et al. 1997). AC Andrew, a Canadian Western Soft White Spring (CWSWS), is reported to have 2-3% lower protein content than Canada Western Red Spring (CWRS) (SeCan 2006). In the present study AC Andrew was observed to have protein concentrations roughly 6.5% lower than CWRS samples. Head to head comparison of samples may be misleading, however, as local climate and plot conditions at the respective growth sites, Carmongay, Alberta and Melita, Manitoba, could be exacerbating differences in study findings. In general, triticale and wheat used in this study showed no difference in protein content but both displayed protein content ~4.4% higher, on average, than corn. According to linear regression analysis on the values reported in Table 4.1, total starch was negatively correlated with protein ($r=0.895$, $P<0.001$), confirming the inverse relationship (Smith et al. 2006).

Unsurprisingly, the highest measured starch content was found to be 71.55% for CMC, 10% higher than for the average wheat (61.5%). Total starch content, for all wheat samples excluding AC Andrew, ranged from 58.31 – 64.20% and fell within expected ranges (Rakszegi et al. 2008). Normally wheat is 10-15% lower in starch than corn (Sosulski and Sosulski 1994). AC Andrew, however, displayed similar patterns of low protein (10.1%) and high starch (68.6%) as observed for CMC. AC Andrew, the most

commonly grown soft white spring wheat, has generated attention of late as potential bioethanol feedstock due to its high yield and desirable starch content (Phelps et al. 2009). For example, Terra Grain Fuels (TGF), a 150 million liter per year ethanol producer in Saskatchewan, has targeted this wheat as preferred feedstock and is offering local farmers incentive to enter into multi-year production contracts for this grain (TGF 2010).

Table 4.2. Protein, total starch, ash and arabinoxylan (AX) content of whole grain samples.

Class	Variety	Protein (%, dwb)	Total Starch (%, dwb)	Ash (%, dwb)	AX (%, dwb)	Amylose (%)
CPSR	S700 PR	14.9	60.61 ± 3.01c-f	1.78	5.25 ± 0.24b-d	28.17 ± 0.28a-g
	AC Crystal	14.9	60.82 ± 0.62b-f	1.79	4.61 ± 0.23f	26.70 ± 1.00d-f
CPSW	AC Vista	15.2	62.39 ± 0.88b-d	1.76	5.38 ± 0.13a,b	28.59 ± 1.06a-f
	HY 475	14.8	61.74 ± 0.41b-e	1.80	4.61 ± 0.14f	30.29 ± 1.22a,b
CWRS	AC Barrie	16.8	58.31 ± 1.87e,f	1.88	4.70 ± 0.22e,f	29.85 ± 1.71a-c
	Superb	16.7	58.40 ± 2.01e,f	1.98	5.06 ± 0.25b-e	30.53 ± 1.77a
CWHWS	Snowbird	14.9	60.93 ± 0.07b-f	1.74	4.96 ± 0.28c-f	26.40 ± 0.69e-g
CWSWS	SWS 162-008	15.6	60.21 ± 1.78d-f	1.86	5.34 ± 0.16a-c	29.15 ± 0.33a-d
	Bhishaj	14.2	60.70 ± 0.21c-f	1.87	5.43 ± 0.18a,b	27.59 ± 1.53b-g
	AC Andrew	10.1	68.60 ± 0.09a	1.90	4.87 ± 0.15d-f	30.16 ± 1.39a,b
CWRW	CDC Falcon	10.7	63.81 ± 0.26b,c	1.65	5.43 ± 0.18a,b	28.33 ± 1.04a-f
	Buteo	11.3	64.20 ± 0.57b	1.59	5.12 ± 0.10b-d	27.16 ± 0.53c-g
CEGP	Hoffmann	15.4	61.63 ± 0.37b-e	1.94	5.10 ± 0.28b-e	26.23 ± 0.24f,g
	Nass	15.4	58.03 ± 1.55f	2.08	5.23 ± 0.30b-d	30.27 ± 1.36a,b
Triticale	Banjo	13.6	62.26 ± 2.90c-d	2.02	5.60 ± 0.21a	28.97 ± 1.36a-e
CMC	Corn	9.9	71.55 ± 0.24a	1.41	—	25.49 ± 0.53g

[a] Values followed by different letters in the same column are significantly different (P<0.05)

Non-starch polysaccharides (NSPs) consist predominantly of arabinose and xylose sugars, collectively known as arabinoxylans (AX), and are found mostly in the bran and endosperm cell walls of cereal grains. AX represents large fractions of both bran and endosperm cell wall content, ranging from 64-69% (dwb) (Ring and Selvendran 1980) of bran and ~88% (dwb) (Mares and Stone 1973) of the endosperm cell wall. Samples included in this study showed subtle variation in whole kernel AX content, ranging from a

low of 4.61 to a high of 5.43% (dwb), consistent with previous reports of 5.3-6.5% AX content for whole grain wheat (Barron et al. 2007). Traditionally AX, also referred to as pentose sugars, have been considered non-fermentable by yeasts (Gong et al. 1981). However, recent studies have cited the use of engineered strains of yeast capable of fermenting D-xylose, the major sugar found in the hemicellulose hydrolyzate, in mixed sugar fermentation schemes (Bertilsson et al. 2009; Bettiga et al. 2008; Govindaswamy and Vane 2010). According to Agu (2006) pentosans, when not hydrolyzed, are known to cause processing problems, especially when processing wheat, which contain higher quantities than other cereals. Possibly representing a boon for wheat based ethanol production, which presently carries significant quantities of undigested xylose through the process, are 'industrial xylose-fermenting strains of *S. cerevisiae* which are now reaching levels of fermentation performance approaching economical feasibility from lignocellulosics' (Hahn-Hagerdal et al. 2007).

4.3.3 Amylose/Amylopectin Content of Starches

Starch granules are composed of two types of alpha-glucans, amylose and amylopectin (Tester et al. 2004b). The ratio of the two polysaccharides varies according to the botanical origin of the starch but cereal grains maintain ranges of 25-28% amylose and 72-75% amylopectin (Hung et al. 2006). Amylose content (Table 4.2) was found to vary significantly among starch samples; ranging from 26.40 – 30.53% among wheat and triticale samples, whereas the CMC sample contained 25.49% amylose. Several studies have reported normal nonwaxy hard and soft wheat cultivars as possessing amylose contents ranging between 26-28% (Hung et al. 2006; Raeker et al. 1998; Zhao et al. 2009), with corn reported to have similar ranges (Wu et al. 2006). The application of

identical analytical methods, as compared to the study of Zhao et al. (2009), suggests discrepancy in findings may be attributable to distinctions in western Canadian cultivars or sample growing conditions. An amylose content greater than 30% was found in Superb, AC Andrew and Nass, indicating an amylopectin content in these cultivars to be less than 70%. CMC was observed to have an amylose content 3% lower, on average, than wheat varieties.

A reduction in amylose content has been positively correlated with enzymatic digestibility and represents the current accepted paradigm for the pattern of α-amylolysis on native starches. Recent fermentation studies consistently report higher ethanol yields on waxy (>95% amylopectin) substrates than non-waxy counterparts (Lee et al. 2001; Wu et al. 2007; Wu et al. 2006; Zhao et al. 2009). Low amylose content wheat has to be viewed as ideal for potential use as bioethanol feedstock given the findings of Wu et al. (2006, 2007) and Zhao et al. (2009). The lowest amylose content grains found in this study to be statistically similar include Snowbird, AC Crystal, Hoffmann, Buteo, Nass and CMC.

4.3.4 Granule Size Distribution

Present research suggests wheat starch has a trimodal distribution of granule sizes (Bechtel et al. 1990; Raeker et al. 1998; Wilson et al. 2006). However, the existence of the smallest C-type granule remains somewhat putative with many authors reporting only the A and B-type granule populations (Ao and Jane 2007; Morrison and Gadan 1987; Peng et al. 1999). A-type granules make up the bulk of starch (~75% by weight), but are fewer in number than the smaller sized B- and C-type granules (~25% by weight) (Ao and Jane 2007). All wheat samples studied here showed a trimodal starch particle size distribution (PSD) and significant variation in A, B and C-type granule content. Fig. 4.1

shows a linear particle size distribution as volume percent for each sample, with a summary of the total volume percentage each granule type assumes in Table 4.3. The typical particle size for A-granules (>10μm) measured in this study occurred for a particle size between 17.4-20.0μm and was distributed approximately symmetrically around this range (Fig. 3.1). Typical sizes for B (<10-5.75μm) and C (<5.75μm)-granule types were 7.6-10.0 and 1.9-3.8μm, respectively. Previous laser-diffraction analysis reported17-20μm and 4-5μm for A- and B-type granules, respectively (Wilson et al. 2006).

Table 4.3. **Granule size distribution of native wheat, triticale and corn starch measured by Differential Scanning Calorimetry (DSC) at 30% (w/v).**

		Volume (%)		
		Type A	***Type B***	***Type C***
Class	**Variety**	**(>10 um)**	**(<10 - 5.75 um)**	**(<5.75 um)**
CPSR	*S700 PR*	62.44 ± 0.067h	11.65 ± 0.020b	25.91 ± 0.076b
	AC Crystal	72.72 ± 0.046b	8.71 ± 0.014h	18.57 ± 0.025h
CPSW	*AC Vista*	67.46 ± 0.477e	10.15 ± 0.176e	22.39 ± 0.196e
	HY 475	67.54 ± 0.092e	9.15 ± 0.044g	23.31 ± 0.054d
CWRS	*AC Barrie*	62.47 ± 0.095h	11.78 ± 0.075b	25.75 ± 0.040b
	Superb	64.15 ± 0.267f	11.07 ± 0.162d	24.78 ± 0.122c
CWHWS	*Snowbird*	61.00 ± 0.076i	12.22 ± 0.020a	26.77 ± 0.054a
CWSWS	*SWS 162-008*	70.23 ± 0.063d	7.16 ± 0.009i	22.62 ± 0.042e
	Bhishaj	72.27 ± 0.046b	9.71 ± 0.029f	18.03 ± 0.0121i
	AC Andrew	71.67 ± 0.030d	9.25 ± 0.014g	19.08 ± 0.017g
CWRW	*CDC Falcon*	63.74 ± 0.351f,g	10.21 ± 0.109e	26.05 ± 0.251b
	Buteo	63.12 ± 0.135g,h	10.29 ± 0.027e	26.59 ± 0.136a
CEGP	*Hoffmann*	71.46 ± 0.127c	8.85 ± 0.023h	19.7 ± 0.013f
	Nass	61.65 ± 0.144i	11.32 ± 0.066c	27.03 ± 0.073a
Triticale	*Banjo*	79.28 ± 0.032a	5.67 ± 0.014j	15.05 ± 0.001j
		(>6 um)		**(<6 um)**
CMC	*Corn*	89.502 ± 1.637		10.498 ± 0.538

[a] Values followed by different letters in the same column are significantly different (P<0.05)
[b] Due to the large differences between wheat and corn, corn has not been included in any statistical analysis

Wheat A-granules ranged from 61 to 72.72% (by volume), slightly lower than reported values, and clearly represent the dominant particle type. C-granules ranged from ~18-27%, representing the second most prevalent particle type and B-granules, the least prevalent type, ranged from ~9-12%. Total composition for small granules (B- and C-type) for hard spring and winter wheat has been reported to range from 37.1-56.2% (mean 47.3%) and 28.5-49.1% (mean 39.9%), respectively (Park et al. 2009). Study results are consistent with these findings, with hard wheat possessing ~35% small particle fraction. Soft wheat has been reported to have small granule composition ranging from 23.1-42.1% (Raeker et al. 1998), and are consistent with study findings of ~29% for the contribution from B and C-granule fractions. Compared to hard spring and winter wheat, which possessed 34.81% small granules, on average, soft wheat appears to have ~6% (v/v) fewer small granules. Banjo, the triticale included in this study, showed higher concentrations of the A-type granule (~80%) with concomitantly lower B and C-type content (~20%). CMC showed a bimodal distribution with the largest particle type assuming close to 90% of the total volume of particles, and possessing a typical particle size between 12-16μm, considerably smaller than that of wheat reported here as ~17-20μm.

The large lenticular A-granules and the small, spherical B-granules have different physical, chemical and functional properties (Dronzek et al. 1972; Kulp 1973; Meredith 1981; Morrison and Gadan 1987; Park et al. 2004; Raeker et al. 1998; Soulaka and Morrison 1985). The two types of granules (A- and B-) differ in their ratio of amylopectin to amylose (Morrison and Gadan 1987; Soulaka and Morrison 1985; Tester and Morrison 1990), and have differing ratios of amylose to bound lipids (Ao and Jane 2007; Raeker et al. 1998). In cereal starches there are small quantities of naturally occurring lipids which

are capable of forming complexes with amylose (Kwasniewska-Karolak et al. 2008). The presence of amylose-lipid complexes negatively influence production of glucose syrups because it reduces water binding and swelling of starch granules, thus impairing the access of amylolytic enzymes (Matser and Steeneken 1998). Liu et al. (2007) studied the in vitro digestibility of A- and B-type granules from soft and hard wheat flours and found higher resistant starch content in the A- type wheat granule as compared to the B-type granule. Several studies have reported higher amylose content in the A-type granule, explaining, in part, their increased resistance to enzymatic hydrolysis (Ao and Jane 2007; Liu et al. 2007).

Samples exhibiting the lowest fraction of A-granule type found in this study include S700PR, AC Barrie, Snowbird, Buteo and Nass, which all show ~40% total volume of particles derived from small (<10μm) granules. Based on the findings of Liu et al. (2007) it appears that B-type granules may contain less resistant starch, due to lower concentrations of amylose, and may therefore yield higher conversion efficiency of starch to fermentable sugars during industrial feedstock preparation. However, studies report a negative correlation between total starch and volume % of small granules (<10μm) (Raeker et al. 1998). Volume % of small granules, was found in this study to have a weak negative correlation with total starch content, ($r=-0.31$, not significant at $P<0.05$), offering some confirmation to the findings of Raeker et al. (1998). In general, according to these findings, as starch content increases, a trait highly amenable to bioethanol production, the volumetric quantity of A-type granules present in the endosperm appears to increase, as small B and C-type granules decrease, a trait not necessarily desirable for end-use as ethanol feedstock.

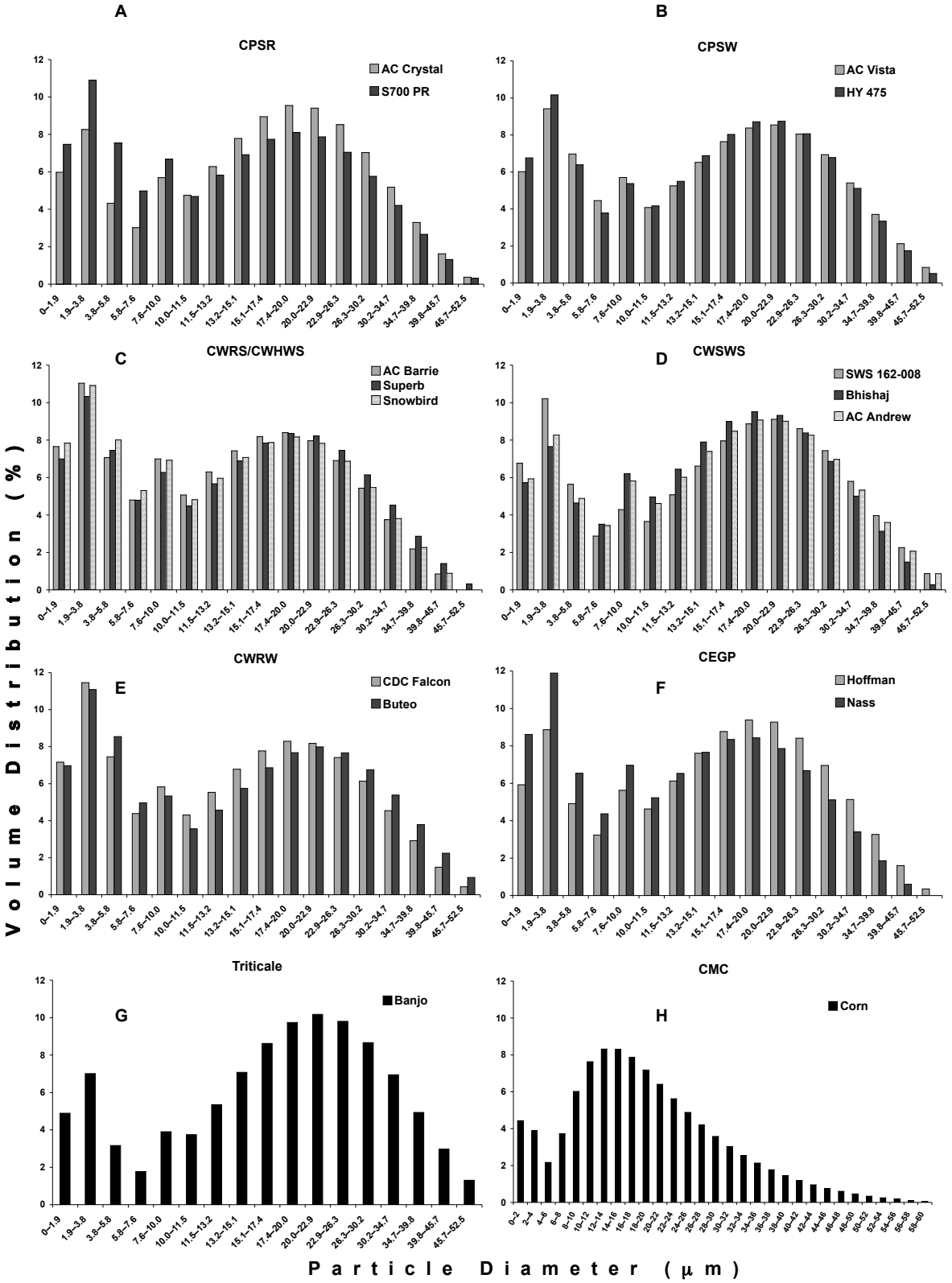

Figure 4.1. Mean starch particle size distribution for wheat, triticale and corn cultivars. (A) CPSR: AC Crystal, S700PR (B) CPSW: AC Vista, HY 475 (C) CWRS/CWHWS: AC Barrie, Superb, Snowbird (D) CWSWS: SWS 162-008, Bhishaj, AC Andrew (E) CWRW: CDC Falcon, Buteo (F) CEGP: Hoffmann, Nass (G) Triticale: Banjo (H) CMC:Corn.

4.3.5 Amylopectin Fine Structure

The two key features of amylopectin fine strucuture are chain length distribution and branching pattern. According to Jenkins and Donald (1995) the currently accepted model for amylopectin structure involves short amylopectin chains forming double helices and associating into clusters. These clusters pack together to produce a structure of alternating crystalline (double helices) and amorphous lamellar composition (amylopectin branch points) (Jenkins and Donald 1995). The branched chains of amylopectin, according to the cluster model of amylopectin proposed by Hizukuri (1986), can be fractionated into B_3, B_2, B_1 and A chains that are described as follows: A-chains; Degree of Polymerization (DP) 6-12, B_1-chains; DP 13-24, B_2-chains; DP 25-36, and B_3-chains; DP>37. A and B_1 chains dominate the distribution, forming double helices, with the longer B_2 and B_3 chains traversing two, three and four clusters (Hizukuri 1986).

Normalized chain length distributions of debranched amylopectin of the wheat samples studied were determined by HPAEC-PAD, and are summarized in Table 4.4 according to peak area ratios defined by Hizukuri (1986). An averaged normalized distribution of all samples is shown in Fig. 4.2, individual sample distributions can be found in Appendix A. No statistical difference was found between samples for each of the peak area ratios, with the exception of DP 13-24, which showed differences between wheat and triticale, but none amongst wheat samples. The averaged chain-length distribution profile calculated from all data suggests the most abundant side-chain had a DP 11, which constituted an average of 7.6% of the total area. According to Fig. 4.2, another pronounced peak occurs at DP 12, constituting, on average, 7.5% of the total area. All samples showed 1st and 2nd peak chain-lengths at DP 11 and 46, respectively. The peak area ratios of DP 6-12, 13-24, 25-36 and DP 37-60 for wheat were 27.13-28.83,

48.47-49.26, 12.44-13.16 and 9.59-10.65%, respectively, and are consistent with previous studies (Hanashiro et al. 1996; Yasui et al. 2005).

During starch gelatinization, starch granular or supramolecular structure is disrupted, resulting in the pattern of enzymatic hydrolysis being 'predomoninently related to the inherent molecular structure of amylopectin' (Zhang et al. 2008b). The inherent molecular structure of amylopectin with a higher amount of branches and shorter chain fraction is not favorable for rapid enzyme digestion (Zhang et al. 2008b). Ao (2008) reports that starch products exhibiting high branch densities, with shorter average chain lengths, showed reductions in rapidly digested starch of up to 30% and concomitant increases in slowly digested starch of up to 20%. The findings of Ao (2008) suggest that longer branch chain lengths of amylopectin may be ideal for a rapidly digestible starch. Waxy starches (>95% amylopectin), indicated as having favorable ethanol conversion performance, tend to have lower proportions of DP 6-12 side chains and higher proportions of DP>35 compared with non waxy starches (Sasaki et al. 2002). With the exception of triticale, there were no statistical differences in the proportion of short branch chains (DP<12) of amylopectin among study samples. Triticale was found to possess ~1% more short chains than the average wheat. These results indicate that crystalline properties of wheat starches considered should be indistinguishable, with the exception of Triticale. When considering the observed thermal and pasting properties, however, discussed in the next sections, there is strong evidence that subtle variation in chain length distribution does have bearing on starch functionality.

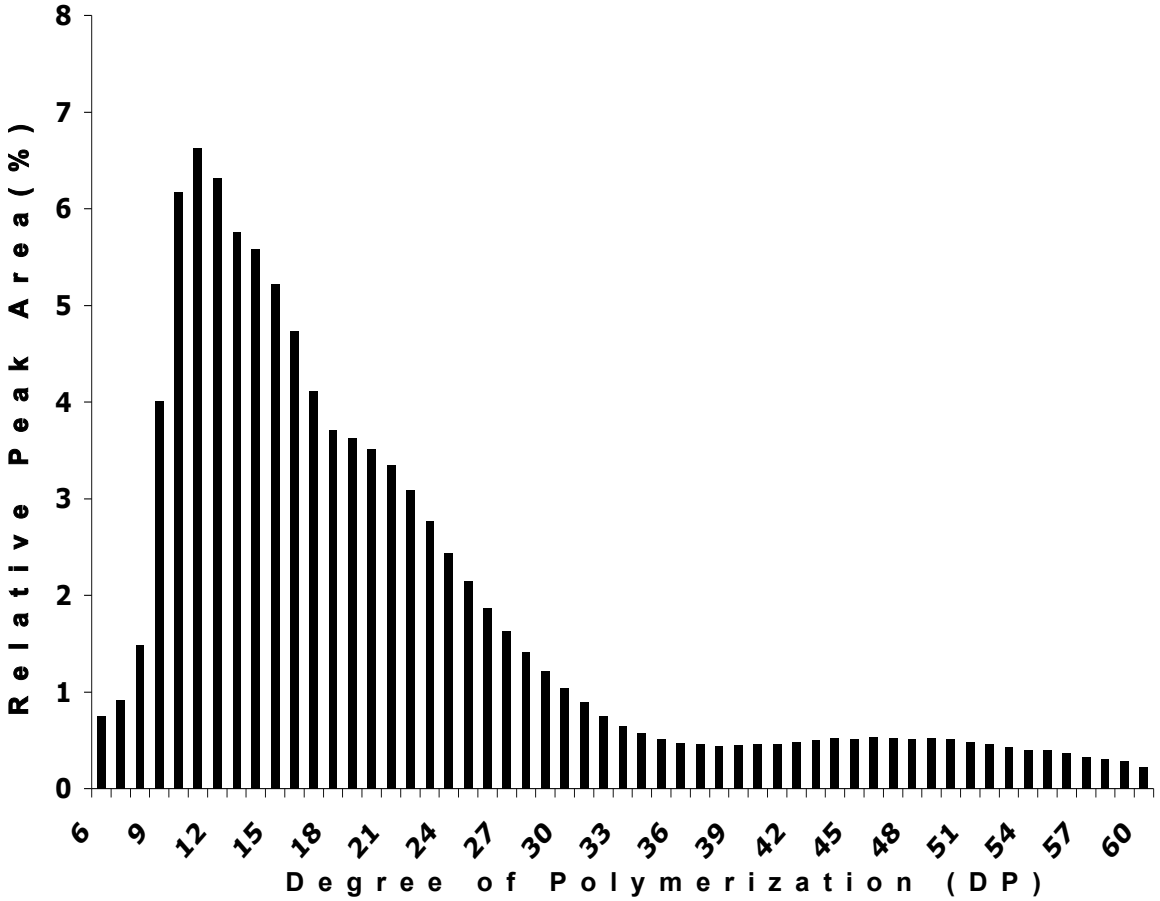

Figure 4.2. Branch-chain length distribution of average wheat amylopectin analyzed by HPAEC-PAD.

Table 4.4. Chain length distribution of the amylopectin fraction, normalized to degree of polymerization (DP) 60 for native wheat and triticale starch.

Class	Variety	Amylopectin Chain Length Distribution (%)			
		DP 6-12[b]	DP 13-24	DP 25-36[b]	DP 37-60[b]
CPSR	*S700 PR*	27.99 ± 1.24	49.08 ± 0.41a	12.44 ± 0.70	10.49 ± 0.15
	AC Crystal	28.20 ± 1.34	48.61 ± 0.01a	13.08 ± 1.07	10.12 ± 0.29
CPSW	*AC Vista*	28.38 ± 0.66	49.22 ± 0.57a	12.57 ± 0.27	9.84 ± 0.18
	HY 475	27.87 ± 0.91	48.95 ± 0.28a	12.66 ± 0.45	10.52 ± 0.17
CWRS	*AC Barrie*	27.90 ± 1.05	49.04 ± 0.29a	12.84 ± 0.26	10.23 ± 0.50
	Superb	28.05 ± 0.98	49.00 ± 0.14a	12.71 ± 0.77	10.26 ± 0.06
CWHWS	*Snowbird*	27.58 ± 1.01	48.98 ± 0.01a	12.80 ± 0.90	10.65 ± 0.13
CWSWS	*SWS 162-008*	27.97 ± 1.51	49.06 ± 0.25a	12.78 ± 1.00	10.20 ± 0.26
	Bhishaj	28.33 ± 0.57	49.12 ± 0.37a	12.98 ± 0.22	9.59 ± 0.01
	AC Andrew	28.83 ± 1.58	48.69 ± 0.13a	12.70 ± 0.97	9.80 ± 0.47
CWRW	*CDC Falcon*	28.24 ± 1.77	48.90 ± 0.10a	12.66 ± 1.17	10.21 ± 0.71
	Buteo	28.42 ± 0.39	49.15 ± 0.64a	12.47 ± 0.06	9.97 ± 0.31
CEGP	*Hoffmann*	28.45 ± 1.32	48.47 ± 0.03a	13.16 ± 1.10	9.93 ± 0.25
	Nass	27.13 ± 1.10	49.26 ± 0.05a	13.12 ± 0.99	10.50 ± 0.16
Triticale	*Banjo*	29.75 ± 1.47	47.62 ± 0.06b	12.60 ± 1.15	10.04 ± 0.25
CMC	*Corn*	-	-	-	-

[a] Values followed by different letters in the same column are significantly different (P<0.05)
[b] No significant differences (P<0.05) were observed among the values by Duncan's Multiple Rang test
[*] CMC was not available for analysis

4.3.6 Thermal Properties

Gelatinization precedes liquefaction in the fermentation process and describes the physical break down of granular starch into solublized, amorphous polymers readily hydrolyzed by α-amylase and amyloglucosidase (AMG), the two enzymes responsible for the conversion of starch to sugar. This irreversible loss of native structure occurs when sufficient energy is applied to break intermolecular hydrogen bonds in the crystalline areas (Rooney and Pflugfelder 1986). Differential scanning calorimetry (DSC) was used to study starch thermal properties of starch:water suspensions (2:3), shown in Table 4.5 and Fig. 4.3. Two endothermic peaks are seen during starch gelatinization, the first representing amylopectin melting and the second the melting of amylose-lipid complexes (Hung et al. 2006). Significant differences were observed for onset temperature (To), peak temperature (Tp), conclusion temperature (Tc) and enthalpy change (ΔH) for both amylopectin and amylose-lipid transition regions. Amongst wheat samples, for the amylopectin transition regions, To, Tp and Tc ranged from 49.53-55.99, 59.72-64.73 and 78.08-87.11^{o}C, respectively. Previous authors have reported To, Tp and Tc, for identical starch:water ratios, as 51.2, 56.0 and 76.0^{o}C (Jenkins and Donald 1998), and are similar to values reported here. CMC displayed a broader transition region, as compared to wheat, with onset, peak and conclusion temperatures occurring at 49.33, 66.12 and 86.74^{o}C, respectively, and are similar to values reported by Jenkins and Donald (1998). Variation in ΔH was observed, with a range of 8.00-11.10 J/g across all samples, and is in accord with the value of 9.0 J/g reported by Jenkins and Donald (1998).

Starch transition temperatures and gelatinization enthalpies by DSC may be related to characteristics of the starch granule, such as the amount of double helical domains (amylopectin) and single helical structures (amylose-lipid complexes) that

unravel and melt during heating of aqueous starch dispersion. Amylopectins with longer branch chains produce more ordered double-helical crystallites, thus requiring higher temperatures to uncoil and dissociate (Song and Jane 2000). The presence of amylose, conversely, lowers the melting (gelatinization) temperature by decreasing crystallinity (Gupta, Bawa and Semwal 2009). Confirming the results of Jane et al. (1992 and 1999), Yuan et al. (1993), Sasaki and Matsuki (1998) and Franco et al. (2002) moderate positive correlation was observed between the percentage of long branch chain (DP>12) amylopectin and peak gelatinization temperature ($r=0.61$, $P<0.05$). However, no correlation between amylose content and onset, peak or conclusion gelatinization temperatures was observed. This is likely due to relatively small differences in amylose content among samples examined in this study. Noda et al. (1998) suggested that gelatinization transition temperatures are more significantly influenced by the nature of amylopectin than the proportion of starch constituted by amylopectin.

Amongst wheat cultivars studied the average peak gelatinization temperature observed was 62.46 ± 1.34^0C, with a range spanning ~4^0C. According to Noda et al. (1998) lower gelatinization temperatures reflects the presence of abundant short amylopectin chains (DP 6-11). Supporting the findings of Noda et al. (1998), the study samples exhibiting the highest fractions of DP 6-12 (Banjo>AC Andrew>Hoffmann>Buteo) (highest to lowest) are ranked in accord with those measured to have the lowest peak gelatinization temperature (Banjo<AC Andrew<Buteo<Bhishaj) (lowest to highest). The average amylopectin short chain content for those samples with peak gelatinization temperature <62^0C (Bhishaj, AC Andrew, CDC Falcon, Buteo, Hoffmann and Banjo) was $28.67\pm0.57\%$, compared to an average of $27.90\pm0.36\%$ for those samples with peak gelatinization temperature >62^0C. In accord with the findings of Noda et al. (1998), the

variation in amylopectin fine structure appears to be the most significant influence on observed gelatinization properties. However, observed difference in amylopectin fine structure are markedly narrow, for example, short chain (DP 6-12) content spans from 27.58±1.01 to 28.45±1.32% amongst samples. It is unclear if such small differences in amylopectin structure could generate a peak gelatinization temperature range of 4^0C amongst samples. In general, the limited variation in gelatinization properties between wheat cultivars is likely due to both the narrow range of observed amylose content and perhaps more significantly, as suggested by Noda et al. (1998), the limited variation in amylopectin fine structure.

Limited research has been performed to elucidate the relationship between gelatinization temperature and industrial fermentation efficiency. From an energy standpoint low gelatinization temperature starch may be favorable as feedstock to produce fermentation-based products due to lower temperatures required to efficiently process the grain. Low gelatinization temperatures, however, are associated with high amylose content starch (Hung et al. 2006; Noda et al. 2002), previously demonstrated to be unfavorable to maximizing ethanol yield. Zhao et al. (2009) found waxy wheat starch to have complete disruption/dissolution of the granule at 70-80^0C, compared to non-waxy cultivars which showed evidence of intact granular structure under hot-stage microscopic visualization for temperatures as high as 90^0C. Wu et al. (2007) also reports that waxy starches easily gelatinize and have concomitantly high conversion efficiency. In regards to bioethanol production the most salient thermal property is likely the point of complete disruption/dissolution, as pointed out by Zhao et al. (2009), and not necessarily traditional transition temperature ranges, as is generally reported. Amylopectin content appears to be

the most influential feature dictating total granular disruption and is perhaps, in this regard, the best predictive metric for ethanol conversion.

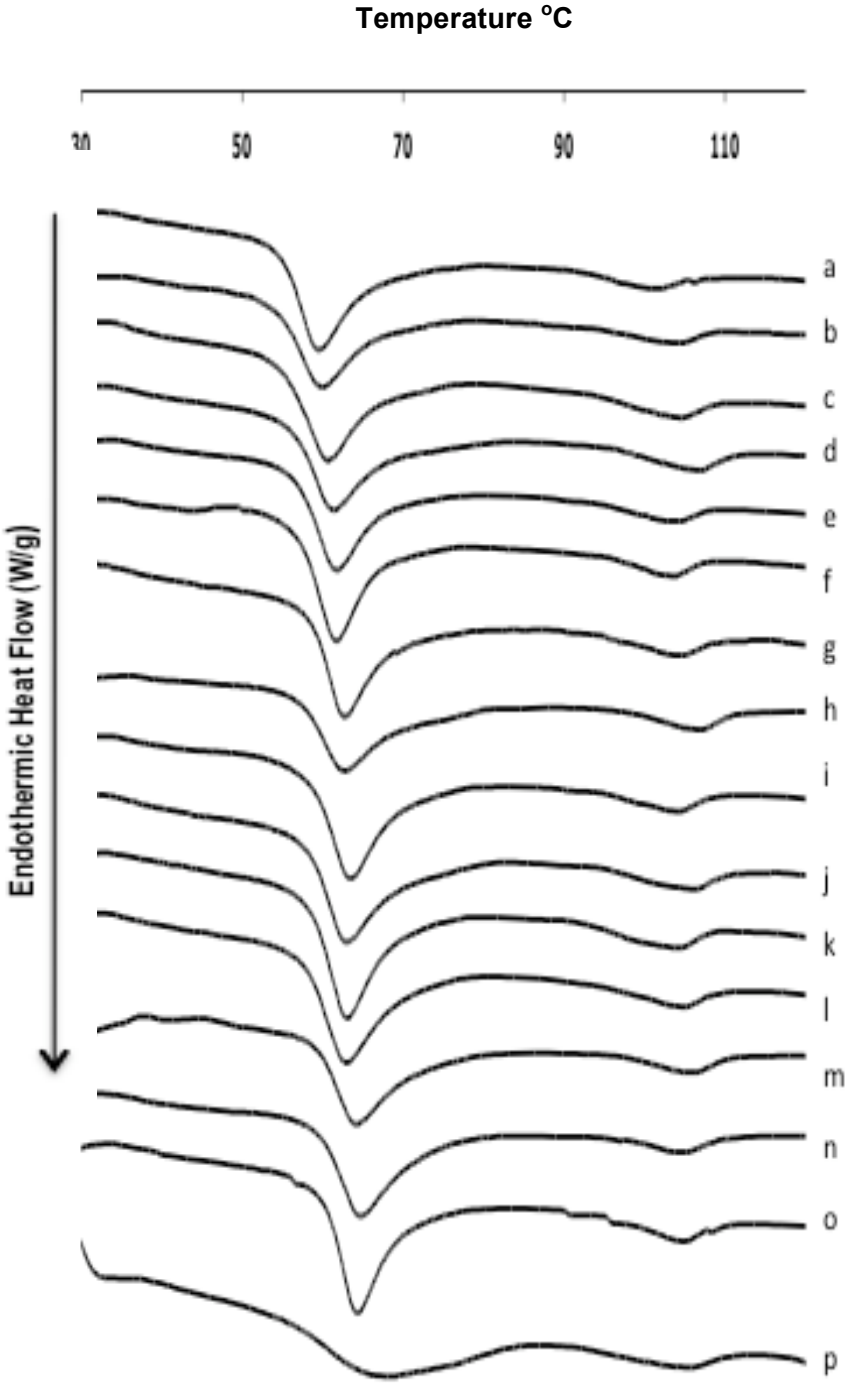

Figure 4.3. Endothermic heat flow for starch samples. a. Banjo b. AC Andrew c. Buteo d. Bhishaj e. CDC Falcon f. Hoffmann g. AC Crystal h. Superb i. AC Vista j. S700PR k. Snowbird l. HY475 m. AC Barrie n. SWS 162-001 o. Nass p. CMC Corn.

Table 4.5 Gelatinization properties described by onset temperature (To), peak temperature (Tp), conclusions temperature (Tc) and enthalpy changes (ΔH) of native wheat, triticale and corn starch measured at 30% (w/w) in water.

		Amylopectin				Amylose-lipid	
Class	Variety	To (°C)	Tp (°C)	Tc (°C)	ΔH (ϑ/γ)	Tp (°C)	ΔH (ϑ/γ)
CPSR	S700 PR	51.62 ± 0.02d,c	62.98 ± 0.13d,e	84.99 ± 0.62a-c	11.10 ± 0.21a	106.45 ± 0.75a	1.82 ± 0.22a,b
	AC Crystal	53.93 ± 0.24a,b	62.56 ± 0.15e	80.40 ± 0.74f-h	9.35 ± 0.48a-d	103.75 ± 1.18c	1.43 ± 0.32a-c
CPSW	AC Vista	51.53 ± 0.24d,c	62.83 ± 0.70d,e	84.64 ± 0.52a-d	10.73 ± 0.38a	104.97 ± 1.09a-c	1.45 ± 0.08a-c
	HY 475	53.02 ± 0.15b,c	63.10 ± 0.16c-e	83.92 ± 0.30b-e	10.37 ± 0.28a,b	104.89 ± 0.38a-c	1.40 ± 0.22a-c
CWRS	AC Barrie	55.14 ± 0.15a,b	63.65 ± 0.88c,d	87.11 ± 0.90a	9.40 ± 1.01a-d	105.73 ± 0.71a-c	1.63 ± 0.20a-c
	Superb	49.94 ± 0.90d	62.76 ± 0.14d,e	84.77 ± 2.40a-d	9.36 ± 0.70a-d	105.82 ± 1.75a-c	1.76 ± 0.13a,b
CWHWS	Snowbird	54.50 ± 0.75a,b	63.00 ± 0.12d,e	78.08 ± 0.15h	8.78 ± 1.51b-d	103.99 ± 0.26b,c	1.87 ± 0.10a
CWSWS	SWS 162-008	50.68 ± 1.05d,c	63.92 ± 0.91b,c	82.96 ± 1.66c-f	9.98 ± 1.24a,b	104.08 ± 0.43b,c	1.41 ± 0.34a-c
	Bhishaj	50.25 ± 0.45d	61.27 ± 0.04g,f	82.11 ± 1.05d-f	8.51 ± 1.33c,d	106.10 ± 1.29a,b	1.73 ± 0.06a,b
	AC Andrew	49.53 ± 2.38d	59.72 ± 0.12h	78.85 ± 0.26h	8.00 ± 0.86d	103.67 ± 0.97c	1.22 ± 0.20c
CWRW	CDC Falcon	50.97 ± 0.89d,c	61.61 ± 0.02f	81.68 ± 1.78e-g	9.54 ± 1.11a-d	104.24 ± 0.11a-c	1.41 ± 0.20a-c
	Buteo	50.84 ± 0.35d,c	60.64 ± 0.01g	81.80 ± 1.95e,f	9.89 ± 0.60a-d	104.74 ± 0.13a-c	1.65 ± 0.31a-c
CEGP	Hoffmann	50.8 ± 1.00d,c	61.68 ± 0.11f	79.01 ± 1.55g,h	9.66 ± 0.48a-d	103.77 ± 1.58c	1.37 ± 0.12b,c
	Nass	55.99 ± 0.00a	64.73 ± 0.50b	85.82 ± 0.73a,b	10.26 ± 0.40a,b	104.75 ± 0.93a-c	1.88 ± 0.06a
Triticale	Banjo	50.68 ± 0.45d,c	59.35 ± 0.11h	80.74 ± 0.60f-h	9.88 ± 0.25a-c	101.09 ± 0.21d	1.17 ± 0.04c
CMC	Corn	49.33 ± 2.32d	66.12 ± 0.11a	86.74 ± 0.91a,b	9.26 ± 0.26a-d	103.74 ± 0.47c	1.64 ± 0.22a-c

[a] Values followed by different letters in the same column are significantly different (P<0.05)

4.3.7 Pasting Properties

The pasting behavior of the starch samples studied was determined using RVA (Rapid-Visco Analyzer) and are displayed in Table 4.6 and Fig. 4.4. The RVA curve describes pasting, a phenomenon following gelatinization, involving granular swelling, exudation of amylose and amylopectin and total disruption of the starch granule. Pasting temperature is the point when the temperature rises above the gelatinization temperature, inducing starch granular swelling and viscosity increases. All wheat starches in this study show an increase in viscosity between 6.4 and 7.0min at a temperature between 92.0 to 95.3^{0}C. CMC and triticale starches, however, show initial viscosity increases at markedly earlier time/temperatures, approximately 5-5.8min and 71.0-84.9^{o}C. The peak viscosity indicates the maximum viscosity reached during the heating and holding cycle and is indicative of the water holding capacity of starch (Gupta et al. 2009). Amongst wheat starch, Snowbird was found to have the highest peak viscosity at 2414cP, roughly 950cP higher than Buteo, found to have the lowest measured peak viscosity. Compared to wheat starch peak viscosity was observed to be relatively high for CMC and triticale, measuring ~2800 and 2400cP, respectively. The degree of RVA breakdown is related to the solubility of starch, and the more soluble the starch, the more it will thin on shearing (Hoseney 1998). Breakdown viscosities amongst wheat ranged from a high of 573 to a low of 213cP, indicating similar breakdown properties between samples. However, CMC was measured to have a breakdown viscosity almost double that of the highest wheat, indicating the starch granules increased susceptibility to shear stress. Total setback involves retrogradation of the starch molecule upon cooling. As the mixture is cooled, re-association between starch molecules, especially amylose, results in the formation of a gel and results in viscosity increases. All samples displayed significant setback viscosity after

cooling with final viscosities for wheat in the range of 2016-3143cP. CMC and triticale had a similar final viscosity measuring approximately 3200cP, noted to be significantly higher than that found for wheat.

Pasting properties of starch are affected by amylose and lipid contents and by branch chain-length distribution of amylopectin (Gupta et al. 2009; Jane et al. 1999). Starches with larger amylose, lipid and phospholipid content have higher pasting temperatures, lower peak viscosity and shear-thinning (breakdown viscosity), and higher setback viscosity (Jane et al. 1999; Zeng et al. 1997). CMC and Snowbird, observed to be the lowest amylose content starches, were also observed to have the highest peak and breakdown viscosities, supporting the claims of Jane et al. (1999) and Zeng et al. (1997). CMC was found to have an amylose content of 25.5% compared to 26.4% for Snowbird, a difference of ~1%. Breakdown viscosity, however, for CMC is found to be nearly double that of Snowbird, and perhaps points to physicochemical differences between wheat and corn starches, in regards to susceptibility to shear stress, beyond amylose content. Setback and final viscosity are reported to increase with amylose content, due to the ability of amylose, more so than amylopectin, to re-associate and form gels upon cooling. Despite their low amylose content, both CMC and Snowbird exhibited higher setback and final viscosity than the high amylose content starches included in this study. In general, a negative correlation between amylose content and peak and breakdown viscosity was observed for study samples; ($r=-0.41$, not significant at $P<0.05$) and ($r=-0.363$, not significant at $P<0.05$), respectively, offering questionable support to the claims of Jane et al. (1999) and Zeng et al. (1997). The effect of amylose content on the properties of retrogradation, as reported by Jane et al. (1999) and Zeng et al. (1997), do not appear to be supported by study findings, however.

Amylose content appears to be more influential to observed pasting properties than amylopectin fine structure. According to Franco (2002) amylopectin with longer branch chains display larger peak viscosity and lower pasting temperatures than shorter chain counterparts. Jane et al. (1999) reports that long chains with DP>50 accelerated retrogradation of amylopectin, whereas the short chains (DP 6~9) retarded it. Differences amongst the pasting properties of wheat cultivars appear to show no correlation with the limited variation in amylopectin side chain fractions, however.

Table 4.6. Pasting properties of native wheat, triticale and corn starch by rapid visco-analysis (RVA) measured at 10% (w/w) in water.

				Viscosity (cP)				
Class	Variety	Peak Time (min.)	Pasting Temp. (°C)	Peak	Trough	Breakdown	Final	Setback
CPSR	*S700 PR*	7.00	92.85	2322.00	1785.96	540.96	2982.96	1197.00
	AC Crystal	6.73	92.10	1890.00	1592.04	297.96	2406.00	813.96
CPSW	*AC Vista*	6.80	92.00	1868.04	1539.96	327.96	2279.04	738.96
	HY 475	6.73	93.65	1961.04	1614.00	347.04	2490.96	876.96
CWRS	*AC Barrie*	7.00	95.00	1740.00	1350.96	390.96	2016.96	666.00
	*Superb**	-	-	-	-	-	-	-
CWHWS	*Snowbird*	6.93	95.25	2414.04	1839.96	573.96	3143.04	1302.96
CWSWS	*SWS 162-008*	7.00	93.60	2060.04	1554.00	506.04	2450.04	896.04
	*Bhishaj**	-	-	-	-	-	-	-
	AC Andrew	6.40	92.00	1575.96	1352.04	224.04	2220.96	869.04
CWRW	*CDC Falcon*	6.80	93.80	1878.00	1529.04	348.96	2327.04	798.00
	Buteo	6.67	92.80	1562.04	1349.04	213.00	2049.96	701.04
CEGP	*Hoffmann*	6.93	90.30	1829.04	1527.96	300.96	2271.00	743.04
	Nass	7.00	93.70	1974.00	1493.04	483.96	2352.96	860.04
Triticale	*Banjo*	5.80	84.85	2397.00	1590.00	807.00	3204.00	1614.00
CMC	*Corn*	5.00	71.00	2804.00	1509.50	1294.50	3224.00	1714.50

[a] No replicates were performed due to insufficient quantity of purified starches, statistical analysis was therefore not possible

* Super and Bhishaj were not available in sufficient quantity to facilitate analysis

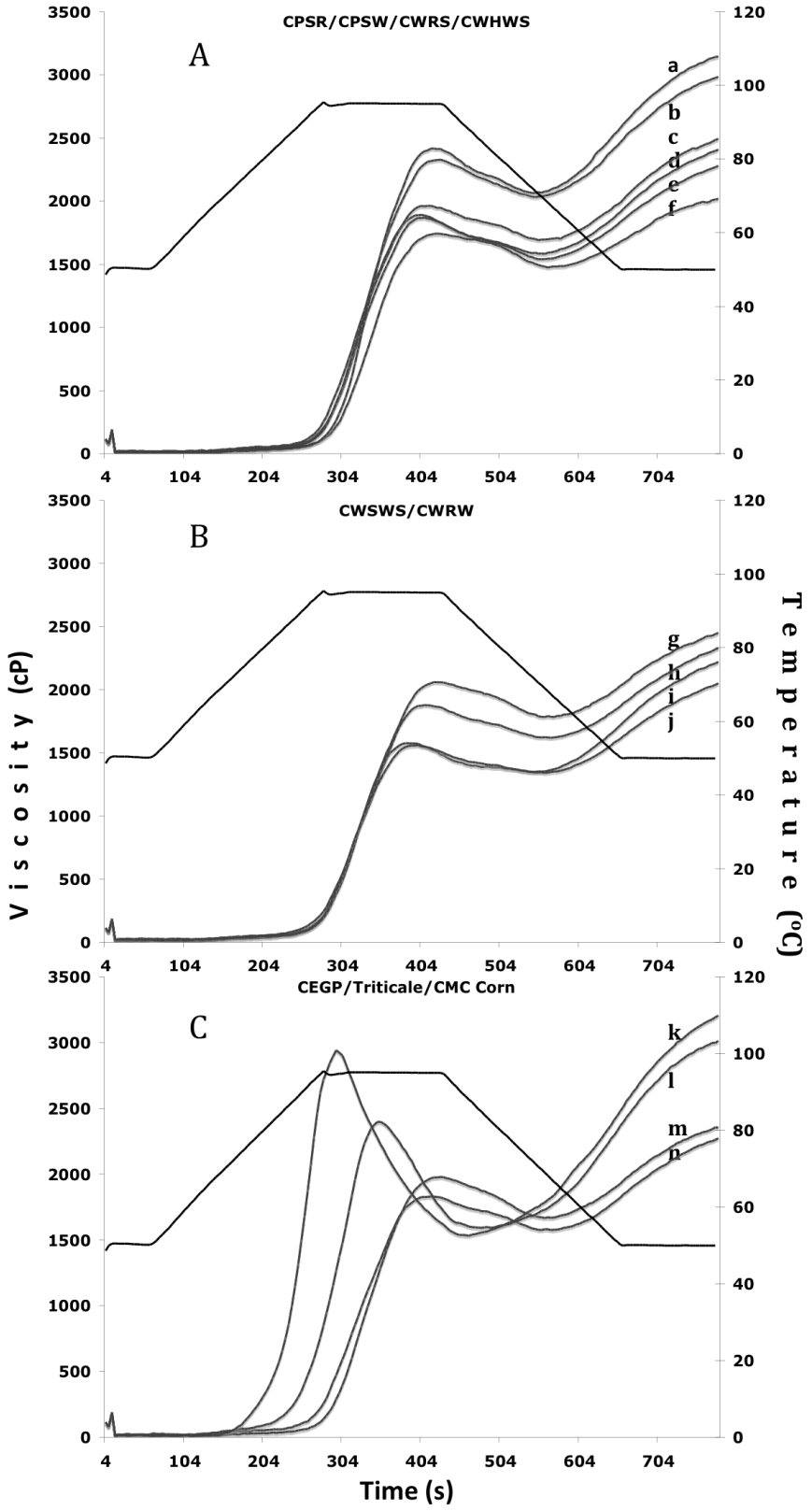

Figure 4.4. RVA pasting curves for starch samples. (A) CPSR/CPSW/CWRS/CWHWS classes, a. Snowbird b. S700PR c. HY 475 d. AC Crystal e. AC Vista f. AC Barrie (B) CWSWS/CWRW classes, g. SWS 162-008 h. CDC Falcon i. AC Andrew j. Buteo (C) CEGP/Triticale/CMC Corn classes, k. Banjo l. CMC Corn m. Nass n. Hoffmann.

Highlighted as desirable for bioethanol production are starches with high solubility (low viscosity after breakdown) and low final viscosity, indicating a decreased ability to retrograde and form resistant complexes to enzymatic degradation upon cooling. A high viscosity in the mash may impair the accessibility of starch to the enzyme and, thus, delay the liquefaction process (Wu et al. 2007). Waxy wheat starch has been described as having lower final viscosity (than non waxy counterparts) (Abdel-Aal et al. 2002; Graybosch 1998; Zeng et al. 1997) and are suggested by Zhao et al. (2009) to be more 'susceptible to breakdown under liquefaction conditions with starch molecules more extensively exposed and more accessible to heat-stable α-amylase.' The highest amylopectin starch included in this study, CMC, displayed a high degree of solubility, twice that of wheat, but also, somewhat inexplicably, the highest observed final viscosity. The wheat samples appear to follow a striking trend in peak, breakdown and final viscosity and are observed to have little relationship to amylose content. Those samples with the highest peak viscosity also displayed the highest viscosity after breakdown and the highest final viscosity. These results indicate that breakdown and setback viscosity varied little between wheat cultivars and suggest that low peak viscosity is perhaps the best indicator of low final viscosity. These findings suggest wheat cultivars possess similar properties of solubility and retrogradation and that selection of wheat with low viscosity after breakdown and setback may be best facilitated by selection of wheat with low peak viscosity. The similarity observed amongst wheat samples in regards to solubility and retrogradation, however, may be due to the narrow range, ~4% , in amylose content amongst starches.

4.3.8 Gelatinization, Liquefaction and Reducing Sugar Profiles

The quantity of reducing sugars present after α-amylase digest is presented in Table 4.7. The starches extracted from different cultivars showed variable susceptibilities to industrial α-amylase attack, mg glucose equiv. ranged from approximately 400 to 500 units per g starch. Those samples yielding the highest quantity of reducing sugars after amylolytic attack are considered to have the greatest number of short chain oligosaccharides present. Conversely, those samples with the lowest reducing sugar yield are considered to have the greatest number of long chain oligosaccharides present. The relative order of hydrolysis, from most to least susceptible, is as follows: HY 475>CDCFalcon >Superb >Bhishaj>AC Barrie>AC Andrew>Hoffmann>Nass >S700PR>AC Crystal >Banjo>AC Vista>SWS 162-008>CMC>Buteo.

Table 4.7. Reducing sugar content of gelatinized starches after hydrolysis with α-amylase.

		Reducing Sugars
Class	**Variety**	**(mg-glucose equiv./g-starch)**
CPSR	*S700 PR*	462.02 ± 15.76b-e
	AC Crystal	456.26 ± 0.83c-e
CPSW	*AC Vista*	450.35 ± 14.88d,e
	HY 475	500.86 ± 0.14a
CWRS	*AC Barrie*	476.31 ± 16.60a-d
	Superb	483.45 ± 13.09a-c
CWHWS	*Snowbird*	458.74 ± 18.91b-e
CWSWS	*SWS 162-008*	441.91 ± 7.57e
	Bhishaj	474.30 ± 19.27a-d
	AC Andrew	473.03 ± 13.83a-d
CWRW	*CDC Falcon*	488.55 ± 5.88a,b
	Buteo	407.27 ± 4.08f
CEGP	*Hoffmann*	467.10 ± 19.12b-e
	Nass	467.05 ± 8.90b-e
Triticale	*Banjo*	450.51 ± 10.54d,e
CMC	*Corn*	408.69 ± 4.74f

[a] Values followed by different letters in the same column are significantly different (P<0.05)

The quantity of reducing sugars present shows no obvious relationship with any of the measured physicochemical parameters of starch, with the exception of amylose content. Fig. 4.5 depicts a positive correlation between reducing sugars and amylose content ($r=0.577$, $P<0.05$). The relationship of liquefied hydrolyzate to the action of amyloglucosidase (AMG), the saccharifying enzyme used to generate fermentable sugars, and fermentation performance, however, remains unclear.

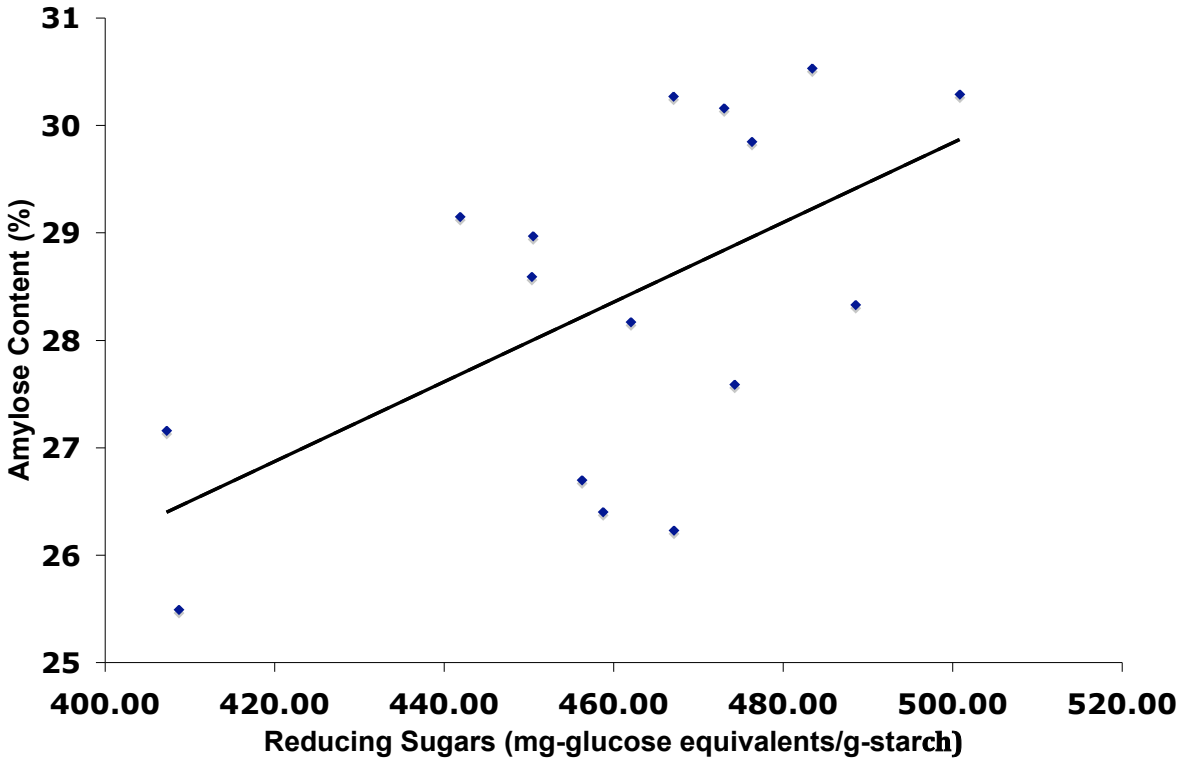

Figure 4.5. Linear correlation between amylose and reducing sugar content.

4.5 Conclusions

This investigation suggests that amylopectin and amylose ratio, granule size, pasting properties and liquefaction performance of different western Canada wheat cultivars were different and depended on cultivar. Amylopectin fine structure, however, was shown to be consistent across measured cultivars, and those properties thought to be

dependant on amylopectin structure, such as thermal properties, displayed statistically significant but subtle variation. The observed similarities for amylopectin structure suggest crystalline properties of cultivars included in this study, when considering their amylopectin fraction, should exhibit similar patterns of enzymatic hydrolysis. In this regard, amylopectin fine structure is likely an unsuitable metric for selection of wheat intended for use as bioethanol feedstock and parameters indicative of total quantity of amylopectin, such as total amylose content, are likely to serve as a more meaningful comparative tool in selection. Amylopectin/amylose ratio displayed significant influence on liquefaction performance and is considered at this time to be the most salient physicochemical property of starch determining susceptibility to enzymatic attack.

Chapter 5: Fermentation Performance of Select Starches Isolated from Western Canadian Wheat

5.1 Introduction

Ethanol yield, perhaps the most important fermentation performance criteria for the fuel ethanol industry, has been shown to be a starch related property of wheat (Kindred et al. 2008; Lacerenza et al. 2008; Zhao et al. 2009). Obviously a wheat cultivar with higher starch content in its grain is desirable because it will provide more ethanol per ton of grain (Zhao et al. 2009). Wheat bran, germ, and outer endosperm proteins, which do not contribute to ethanol yield, are carried through the process (Sosulski and Sosulski 1994), decreasing per tonne ethanol yield as their quantities increase. Several strategies to increase starch composition in bioethanol feedstock are under investigation, including development of high yield cereal grain hybrids (Koutinas et al. 2004; Wu et al. 2006), preprocessing strategies to debran wheat (Sosulski et al. 1997), and advanced agricultural practices to minimize protein content in the mature grain (Kindred et al. 2008).

Elite genotypes for ethanol production have been described as having rapid liquefaction characteristics, low viscosity during liquefaction, high fermentation speed and high fermentation efficiencies (Wu et al. 2007). The parameters of whole grain systems that yield the aforementioned conditions have not been elucidated, nor has the relationship of these conditions to the physicochemical attributes of contained starches. The overall objectives of the research described in this chapter include an assessment of fermentation performance of pure starch systems and, drawing upon the physicochemical data presented in Chapter 3 of this thesis, an analysis of the unique physicochemical parameters of starch that correlate with high ethanol conversion efficiencies.

5.2 Materials & Methods

Material and methods for fermentation performance analysis is described in sections 3.2.1 to 3.2.7 of this thesis. Liquefaction performance was performed according to section 3.1.10 Statistical analysis was performed according to section 3.3.

5.2.1 Starch Liquefaction Performance

The performance during starch hydrolysis was evaluated based on the maltose production and the concomitant liquefaction yield (Y_M) (g-maltose/g-substrate) for each substrate. The liquefaction yield was calculated according to equation 5.1, where M indicates the maltose concentration. The initial maltose concentration for all samples was assumed to be zero.

$$Y_M = \frac{(M_{final} - M_{initial})\ (g)}{\text{Substrate (g)}} \qquad (5.1)$$

Also considered as an aspect of liquefaction performance is the generation of glucose (Y_G) (g-glucose/g-substrate), maltotriose (Y_{DP3}) (g-maltotriose/g-substrate) and maltotetrose (Y_{DP4}) (g-maltotetrose/g-substrate), and are reported similarly to maltose concentration, with initial values assumed as zero.

5.2.2 Fermentation Performance

The SSF performance was evaluated based on the ethanol yield ($Y_{E/S}$) (g-ethanol/g-substrate), obtained using Equation 5.2. The major metabolic by-products of fermentation, glycerol and acetic acid, were also monitored and evaluated based on their respective yields, glycerol yield ($Y_{G/S}$) (g-glycerol/g-substrate) and acetic acid yield ($Y_{A/S}$) (g-acetic acid/g-substrate), obtained using Equation 5.3 and 5.4, respectively.

$$Y_{E/S} = \frac{\text{Ethanol Produced (g)}}{\text{Substrate (g)}} \quad (5.2)$$

$$Y_{G/S} = \frac{\text{Glycerol Produced(g)}}{\text{Substrate (g)}} \quad (5.3)$$

$$Y_{A/S} = \frac{\text{Acetic Acid Produced (g)}}{\text{Substrate (g)}} \quad (5.4)$$

Conversion efficiency was calculated as actual ethanol yield ($Y_{E/S}$) divided by theoretical ethanol yield, reported as 0.5672 g-ethanol/g-starch (Wu et al. 2007), observed in Equation 5.5.

$$\text{Conversion Efficiency} = \frac{Y_{E/S}}{Y_{\text{Theoretical}}} \times 100 \quad (5.5)$$

5.2.3 Kinetics of Yeast Growth

The generation time (also called doubling time) for *S. cerevisiae* was calculated and compared between substrates. The microbial growth rate (μ) was calculated by linear regression of the logarithmic number of yeast cell (Log N) during the exponential phase of growth (Equation 4.6).

$$\text{Slope} = \frac{\mu}{2.303} \quad (5.6)$$

The generation time (g) was calculated according to equation 4.7.

$$\mu = \frac{ln\ 2}{g} \quad (5.7)$$

5.2.4 Bioavailability of Fermentable Sugars

The glucose equivalents (GE) for maltotetrose (DP4), maltotriose and maltose were each calculated for initial (T_{0h}) and final (T_{24h}) time points during fermentation, according to Equation 5.8, 5.9 and 5.10, respectively, where M indicates mass of material in g/g-starch. The summation of these values, for the respective time points, were used to calculate the bioavailability of saccharified and fermented starch and represents the percent total sugar (expressed as GE) consumed during fermentation, as observed in Equation 5.11.

$$GE_{DP4} = \frac{M_{DP4}}{666\ \text{g-DP4/mole}} * \frac{4\ \text{glucose molecules}}{1\ \text{DP4 molecule}} * \frac{180.16\ \text{g-glucose}}{\text{mole}} \quad (5.8)$$

$$GE_{maltotriose} = \frac{M_{maltotriose}}{504.44\ \text{g-DP4/mole}} * \frac{3\ \text{glucose molecules}}{1\ \text{DP4 molecule}} * \frac{180.16\ \text{g-glucose}}{\text{mole}} \quad (5.9)$$

$$GE_{maltose} = \frac{M_{maltose}}{342.20\ \text{g-DP4/mole}} * \frac{2\ \text{glucose molecules}}{1\ \text{DP4 molecule}} * \frac{180.16\ \text{g-glucose}}{\text{mole}} \quad (5.10)$$

$$\text{Bioavailability} = (\text{Total } GE_{T0h} - \text{Total } GE_{T24h}) / \text{Total } GE_{T0h} \quad (5.11)$$

$$\text{Total } GE_{T0h} = SUM_{(T0h)}(GE_{DP4,maltotriose,maltose}) + glucose_{(T0h)} \quad (5.11a)$$

$$\text{Total } GE_{T24h} = SUM_{(T24h)}(GE_{DP4,maltotriose,maltose}) + glucose_{(T24h)} \quad (5.11b)$$

5.3 Results & Discussion

5.3.1 Starch Liquefaction Performance

Liquefaction is the preliminary step in industrial ethanol fermentation schemes, as intact starch is inaccessible to yeast and must be converted to soluble sugars prior to fermentation. Liquefaction involves the hydrolysis of α-(1,4)-glucans by amylolytic enzymes, lowering the slurry viscosity, releasing disaccharides such as maltose, for posterior saccharification and fermentation (Abd-Aziz et al. 2001). A profile of the fermentable sugars generated after liquefaction with porcine pancreatic α-amylase at 10% solids (w/v) substrate concentration is shown in Table 5.1. Analysis was carried out via molecular weight determination using a high performance size exclusion chromatography (HPSEC-MALS) system.

Table 5.1. Yield of fermentable sugars generated after 120 min digest with porcine pancreatic α-amylase analyzed via molecular weight determination using HPSEC-MALS.

Class	Variety	Y_G (g/g-starch)	Y_M (g/g-starch)	Y_{DP3} (g/g-starch)	Y_{DP4} (g/g-starch)
CPSR	S700 PR	0.036	0.342	0.297	0.043
CWHWS	Snowbird	0.033	0.346	0.294	0.040
CWSWS	AC Andrew	0.038	0.366	0.298	0.029
CWRW	CDC Falcon	0.036	0.342	0.309	0.061
	Buteo	0.034	0.354	0.292	0.037
Triticale	Banjo	0.036	0.338	0.299	0.045
CMC	Corn	-	-	-	-

* Glucose yield (Y_G), maltose yield (Y_M,) maltotriose yield (Y_{DP3}), maltotetrose yield (Y_{DP4})
* CMC was not available for analysis
* Samples were not run with replicates therefore statistical analysis was not available

The production of maltose during α-amylolytic attack, considered a meaningful metric in the assessment of liquefaction performance (Das Neves 2006), was found to range from 0.338 to 0.366 g-maltose/g-starch. Maltose was found to be the dominant

sugar after liquefaction for all samples, representing an average of 35.1% (weight basis) of the total sugars present, as evidenced in Table 5.1. Maltotriose and larger dextrans (>DP4) were also present in large quantities, representing an average of 32.7 and 23.8% (weight basis) of the total sugars present, respectively, after liquefaction. The average maltose concentration across all samples was 0.348±0.010 g-maltose/g-starch with the range spanning 0.028 g-maltose/g-starch.

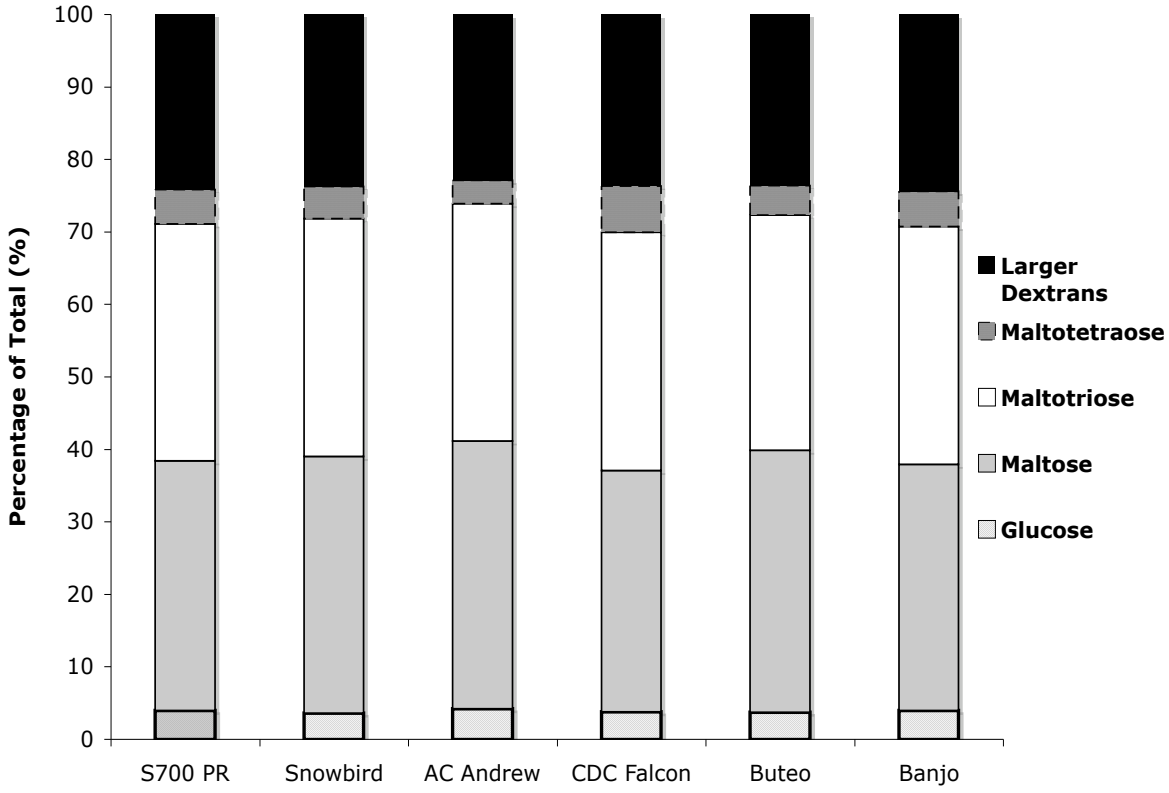

Figure 5.1. Profile of soluble sugars (glucose, maltose, maltotriose, maltotetrose) and larger dextrans present after 2 hr incubation of gelatinized starch with porcine pancreatic α-amylase.

Maltose is the most significant sugar in the process regulation of SSF because it is substrate not only for amyloglucosidase (AMG), the enzyme responsible for saccharification, but also for the yeast (Montesinos and Navarro 2000b). According to Das Neves (2006), increasing quantities of maltose in the liquefied product are thought to

yield concomitant increases in glucose during saccharification. However, consensus on the role of maltose in saccharification, and the relative benefits of having high proportions of this sugar in the wort, has not been reached. According to Montesinos and Navarro (2000), maltose has a repressing effect on AMG reducing its activity by twenty fold, which is lifted only after yeast have begun consumption of maltose, generally resulting in rapid ethanol production. The patterns of hydrolysis evidenced in Fig. 5.1 suggest marginal differences between samples in regards to the action of α-amylase on gelatinized material. Based on the evidence of Das Neves (2006) the equivalent liquefaction performance observed between samples should yield equivalent glucose concentrations upon saccharification. These small differences observed for liquefaction performance do not offer explanation, however, for significant differences observed in saccharification and fermentation performance, discussed in the proceeding section.

5.3.2 Fermentation End-Product Synthesis

Ethanol, glycerol and acetic acid were the main accumulated products in the liquid phase during growth of *S. cerevisiae* in batch culture on hydrolyzed starch. Lactic acid was measured but not detected, indicating no contamination with lactic acid producing microorganisms. End-product results from the fermentation experiments are described in Table 5.3. The substrates used as fermentation feedstock generated two patterns of ethanol production after 24 h SSF, evidenced in Fig. 5.3. CMC and Buteo yielded 0.550±0.015 and 0.542±0.013 g-ethanol/g-starch, respectively, with conversion efficiencies ranging from ~95-97% (Table 5.2). The remaining 5 substrates produced roughly 10% less total ethanol, with yields between 0.486 – 0.502 g-ethanol/g-starch, and conversion efficiencies between ~85-88%. In industry, the ethanol yield that is calculated based on the total sugar feeding into the fermentation system without deduction of the residual sugar can be

as high as 90-95% (Ingledew 1999; Thomas and Ingledew 1990; Wu et al. 2006). Total ethanol yields are likely inflated in this study by a small percent, as enzyme preparations for α-amylase and AMG contained quantities of free sugars, specifically glucose, which could not be controlled for. As the study is comparative, and all samples had equivalent background sugars, adjusted values are not reported but are noted to fall within conversion efficiency ranges reported by Ingledew (1999), Thomas and Ingledew (1990) and Wu et al. (2006), for the highest yielding samples.

Table. 5.2. Conversion efficiencies of various starch samples to ethanol during SSF using *S. cervisiae.*

Class	Variety	Conversion Efficiency (%)
CPSR	S700 PR	88.14
CWHWS	Snowbird	88.42
CWSWS	AC Andrew	87.56
CWRW	CDC Falcon	85.64
	Buteo	95.57
Triticale	Banjo	85.87
CMC	Corn	97.00

*values calculated according to Eqn. 5.5

Table 5.3. Final end-product yield for Ethanol ($Y_{E/S}$), Glycerol ($Y_{G/S}$) and Acetic Acid ($Y_{A/S}$) after 24 hr batch SSF.

Class	Variety	$Y_{E/S}$[a] (g/g-starch)	$Y_{G/S}$[b] (g/g-starch)	$Y_{A/S}$[b] (g/g-starch)
CPSR	S700 PR	0.500 ± 0.023*b*	0.057 ± 0.003	0.012 ± 0.007
CWHWS	Snowbird	0.502 ± 0.016*b*	0.057 ± 0.004	0.009 ± 0.000
CWSWS	AC Andrew	0.497 ± 0.011*b*	0.059 ± 0.002	0.005 ± 0.010
CWRW	CDC Falcon	0.486 ± 0.016*b*	0.057 ± 0.003	0.006 ± 0.004
	Buteo	0.542 ± 0.013*a*	0.057 ± 0.008	0.006 ± 0.003
Triticale	Banjo	0.487 ± 0.026*b*	0.055 ± 0.003	0.005 ± 0.001
CMC	Corn	0.550 ± 0.015*a*	0.059 ± 0.004	0.005 ± 0.001

[a] Values followed by different letters in the same column are significantly different (P<0.05)
[b] No significant differences (P<0.05) were observed among the values by Duncan's Multiple Rang test
[*] Reported values are averages of all replicates (n=3) and their standard deviations

Ethanol production over the entire 24 h batch SSF period, displayed in Fig. 5.3, suggests *S.cerevisiae* entered exponential growth at approximately 1 h after inoculation and remained in exponential growth until approximately 5 h after inoculation, and remained in near exponential growth for most samples until approximately 8 h after inoculation. Ethanol, a primary metabolite, is directly tethered to fermentative cell growth, therefore the majority of ethanol production occurs during this exponential phase of growth. In this study, the quantity of ethanol produced during exponential growth represents ~95% of the final yield, the remaining 5% produced in stationary phase.

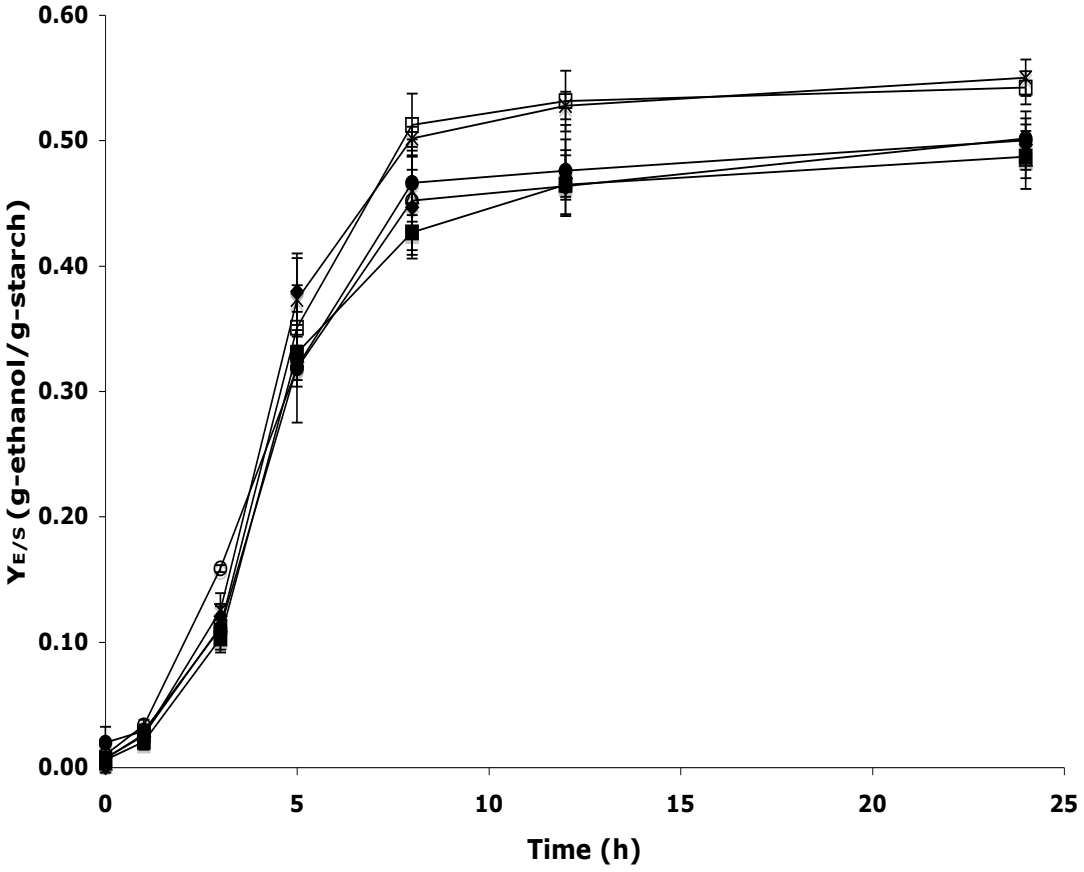

Figure 5.3. Time course of ethanol generation for various starch substrates during SSF. Symbols: ◆, AC Andrew; ■, Banjo; ❑, Buteo; ✕, Corn; Δ, Falcon; S700PR, ●; Snowbird, ❍. Error bars represent the standard deviation for replicates (n=3).

Glycerol production ranged from 0.055-0.059 g/g-starch and represents, on average, 10-11% (w/w) of ethanol yield. Glycerol, reportedly produced at a level of about 1.0% (w/v) for most industrial ethanol fermentations (Bai et al. 2008), helps maintain intracellular redox balance and is a response to osmotic stress. Glycerol production in the study corresponds well with cited literature, when assuming most industrial ethanol facilities achieve an ethanol yield ranging from 12-13% (v/v) (9.5-10.3% (w/v)), and means glycerol yield is ~10% (w/w) of ethanol yield. Glycerol production lowers ethanol

yield and is considered unfavorable (Wang et al. 2007). Achieving lower levels of glycerol production through enhanced environmental control, in regards to pH, temperature and dissolved oxygen, is an area worthy of further investigation. Reported glycerol production appears highly uniform, indicating similar levels of osmotic stress between samples. All study samples follow a similar trend in glycerol production, as displayed in Fig. 5.4. Over ~95% of glycerol yield is reached by the end of exponential growth, occurring at approximately 8 hr after the start of fermentation, and increases only marginally between 8-24 h.

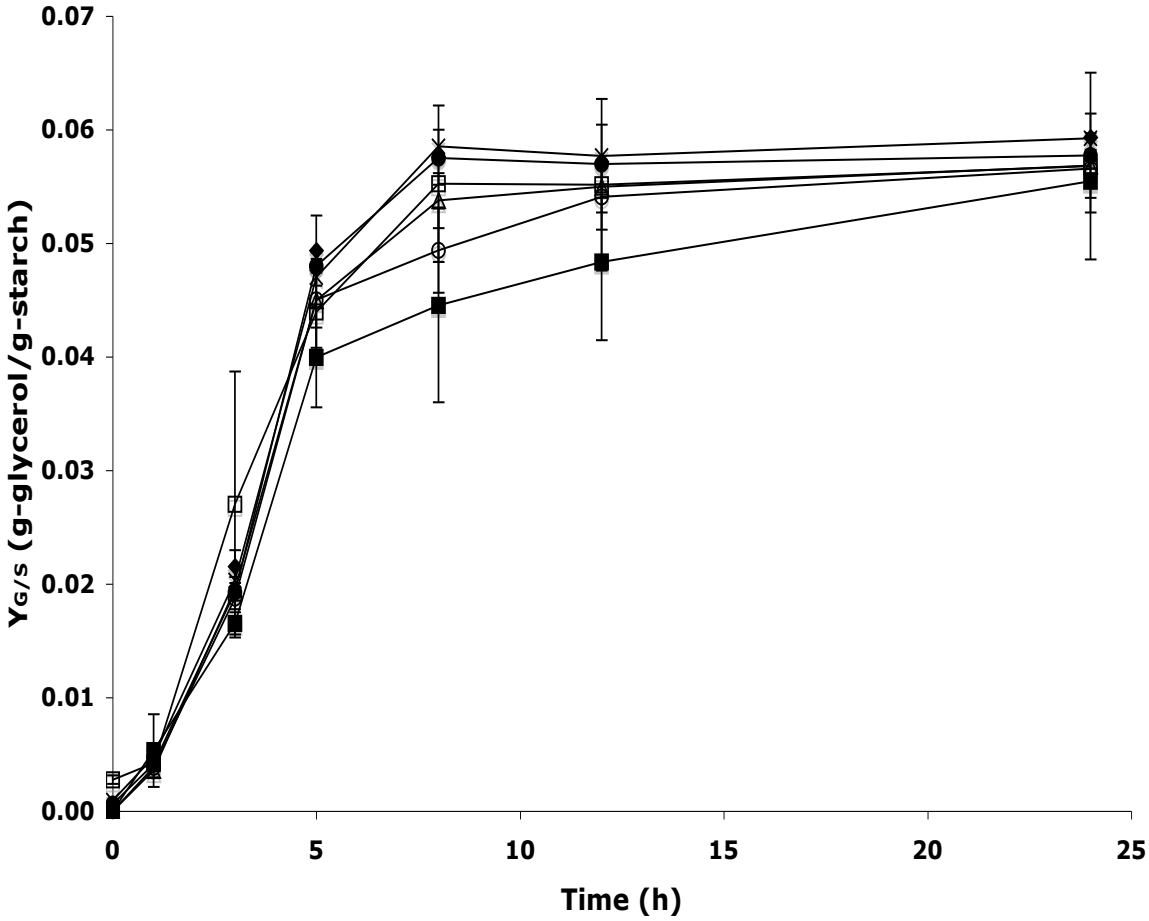

Figure 5.4. Time course of glycerol generation for various starch substrates during SSF. Symbols: ◆, AC Andrew; ■, Banjo; ❑, Buteo; ✕, Corn; Δ, Falcon; S700PR, ●; Snowbird, ❍. Error bars represent the standard deviation for replicates (n=3).

Other by-products such as organic acids and higher alcohols, mainly propanol and butanol, are produced at much lower levels (than glycerol) (Bai et al. 2008). Acetic acid is a by-product of ethanol production and inhbits fermentation in an exponential way (Krisch and Szajani 1997). Acetic acid was produced in very low levels ~1% (w/w) of ethanol yield and is thought to be formed from the oxidation of acetaldehyde (Eglinton et al. 2002) or contamination with ethanol consuming *acetobacters* (Krisch and Szajani 1997). Contamination with *acetobacter*, however, in the strict anaerobic culture

employed in this study is not possible, as this microorganism is aerobic. There is the possibility, however, of contamination occurring post-fermentation, during sample preparation for analysis. Fig. 5.5 suggests that yeast cell growth and acetic acid production are not tightly coupled, as acetic acid was produced, for most samples, over the entire 24 h batch culture. The samples exhibit a wide range of production, from 0.012 – 0.005 g-acetic acid/g-starch and suffer large standard deviations. The low quantities of acetic acid, ~0.01% (w/v), were likely on the lower level of resolution for the HPLC employed, which was calibrated to industrial concentrations, making accurate detection problematic and resulting in large observed errors.

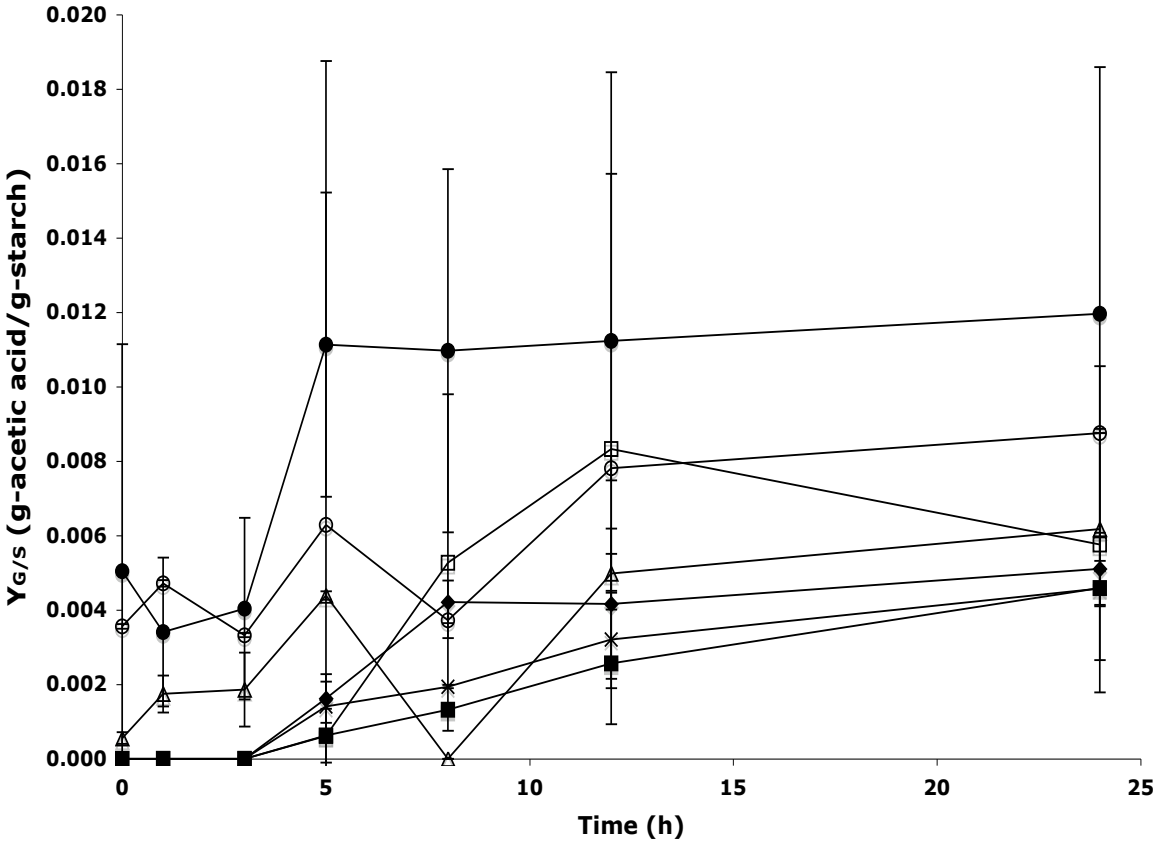

Figure 5.5. Time course of acetic acid generation for various starch substrates during SSF. Symbols: ◆, AC Andrew; ■, Banjo; ❑, Buteo; ✕, Corn; Δ, Falcon; S700PR, ●; Snowbird, ❍. Error bars represent the standard deviation for replicates (n=3).

5.3.3 Kinetic Parameters

The kinetic parameters of yeast cell growth were established for each of the seven substrates. According to Fig. 5.6, during the first hour of SSF the yeast cell's remained in lag phase, after which exponential growth commenced for approximately 7 h. Roughly 8 h after inoculation the cells entered stationary phase, indicating nutrient depletion in the fermentation broth. Subtle increases in cell density after cessation of exponential growth have been reported. Authors have linked residual cell growth to the consumption of ethanol by yeast at the end of starch saccharificaiton (Das Neves 2006; Fujii et al. 2001). In this study, however, no increases in cell density are observed after 12 h for the majority of samples. In fact, yeast cell density appears to decrease after 12 h, likely a result of cell death and flocculation, possibly skewing OD_{600} measurements downwards despite vigorous mixing. The studies of Das Neves (2006) and Fujii et al. (2001) do not maintain strict anaerobic conditions during fermentation and rely on yeast propensity for fermentation in the presence of ample glucose. In oxidative conditions, however, yeast are able to switch to an ethanol consuming metabolism in the absence of preferred nutrients (Yonsel et al. 2007), explaining the observed post-fermentative shift. The strict anaerobic conditions maintained here likely prevented a diauxic shift to an ethanol consuming metabolism after starch saccharification, and in the absence of a nutrient source cell death ensued. Several samples, however, did show evidence of continued cell growth after 12 h, perhaps indicating incomplete nitrogen purging and residual oxygen content. Further work is recommended to clarify the implications of this finding.

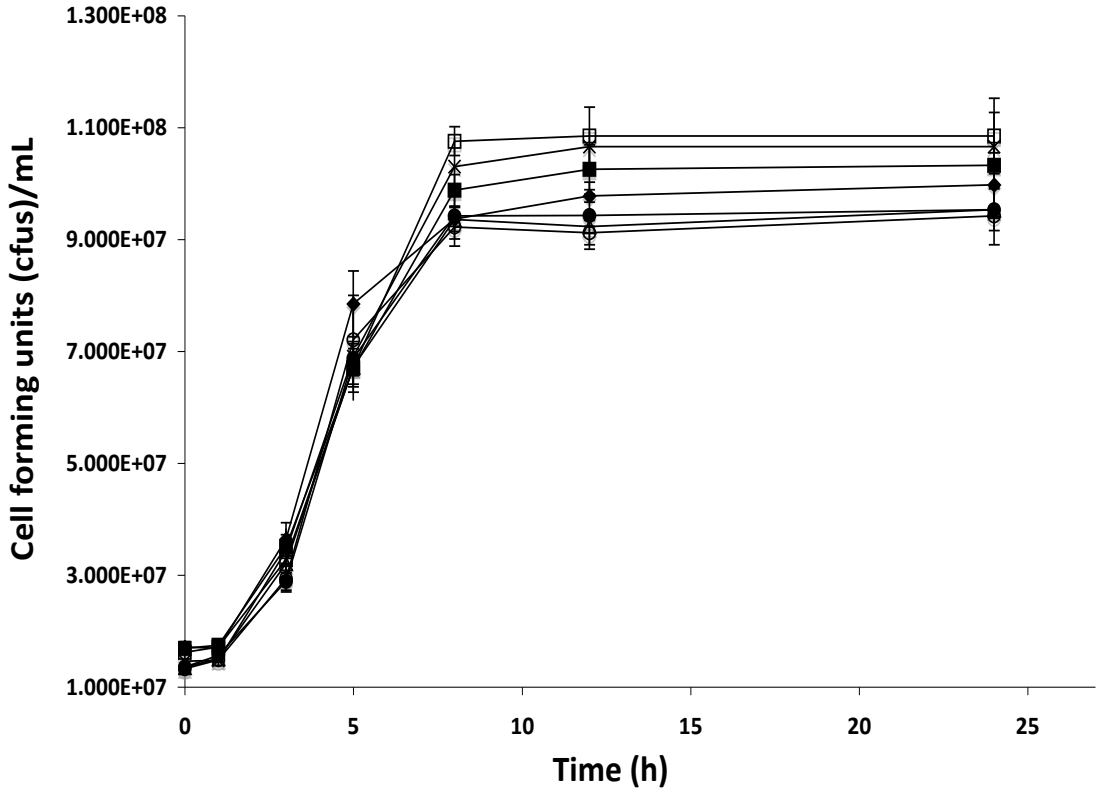

Figure 5.6. Time course of yeast cell growth for various starch substrates during SSF. Symbols: ◆, AC Andrew; ■, Banjo; ❑, Buteo; ✕, Corn; Δ, Falcon; ●, S700PR; ❍ , Snowbird. Error bars represent the standard deviation for replicates (n=3).

The yeast specific growth rate, $\mu(h^{-1})$, was calculated from the slope of the linear dependence of yeast cell number logarithm (log N) on the fermentation time of culture media (h) during an exponential phase of the growth, and substituted into Eqn. 5.6. The generation time (g) for yeast on the different substrates was calculated using Eqn. 5.7, and indicates the average time required for yeast cells to complete one cell cycle. No statistical difference between samples was found for either specific growth rate, μ, or generation time, g, indicating yeast cell growth was similar on each of the substrates. The specific growth rate and doubling time for yeast cells was found to average 0.37±0.02 h^{-1}

and 1.88±0.10 h , respectively, and supports previous findings during SSF on fermentable sugars (Das Neves et al. 2007).

Specific ethanol production rate (Q_p) during exponential growth was calculated and compared between substrates. Ethanol production was graphed against time for the exponential phase of growth and the resultant values (g-ethanol L^{-1} h^{-1}) graphed against yeast cell number (cfus mL^{-1}). The slope of the resultant line was taken as specific productivity (μg-ethanol Million cfu^{-1} h^{-1}). The average specific ethanol production rate was found to be 13.31±1.40 μg-ethanol $Mcfu^{-1}$ h^{-1}, with no statistical differences found between samples, indicating identical ethanol production performance during exponential growth on the respective substrates.

It is unlikely that observed differences in total ethanol production are due to different specific ethanol production rates or yeast growth kinetics during exponential growth, as both parameters were found to be statistically similar between samples. It appears, however, that certain samples remained in exponential phase for a longer period, thus generating increased densities of yeast. Both CMC and Buteo were found to produce higher densities of yeast cells than other samples, following a pattern similar to ethanol production. CMC and Buteo produced ~$1.1x10^8$ cfus/mL after 12 h of fermentation, roughly 10% more cells than were produced in the remaining samples, which averaged ~$9.6x10^7$ cfus/mL in the same time period. For each of the substrates studied specific growth rate begins to slow after 5 h and reaches near zero values after 8 h (Fig. 5.6). Growth rate appears to slow differentially between samples, however, with some remaining in near exponential phase up to the 8 h time point, indicating greater quantities of sugars available to sustain growth.

Table 5.4. Kinetic parameters of exponential yeast cell growth described by specific growth rate (μ) and generation time (g) with concomitant specific ethanol production (Qp).

Class	Variety	μ^a (h^{-1})	g^a (h)	$Q_p{}^a$ (μg-ethanol Million cfu^{-1} h^{-1})
CPSR	S700 PR	0.37 ± 0.01	1.88 ± 0.08	11.91 ± 1.27
CWHWS	Snowbird	0.37 ± 0.00	1.87 ± 0.02	13.33 ± 2.16
CWSWS	AC Andrew	0.38 ± 0.01	1.81 ± 0.03	12.66 ± 0.44
CWRW	CDC Falcon	0.37 ± 0.03	1.86 ± 0.13	12.42 ± 0.96
	Buteo	0.35 ± 0.00	2.00 ± 0.01	14.84 ± 0.76
Triticale	Banjo	0.35 ± 0.02	2.00 ± 0.09	13.13 ± 1.17
CMC	Corn	0.37 ± 0.00	1.80 ± 0.07	14.43 ± 0.96

[a] No significant differences ($P<0.05$) were observed among the values by Duncan's Multiple Rang test
[*] Reported values are averages of all replicates (n=3) and their standard deviations

5.3.4 Fermentable Sugar Utilization

Time-course profiles of glucose, maltose, maltotriose and maltotetrose during SSF, for all substrates, are shown in Fig. 5.7, with concomitant ethanol production. The high glucose concentrations at the beginning of the SSF process were likely caused by the rapid action of AMG on the liquefied starch. In fact, it appears that AMG was able to convert the majority of wort polysaccharides into glucose before the first sample point was drawn, a period ranging from 5-15 min. At the beginning of fermentation it appears that maltose, maltotriose and DP4 concentrations are found to collectively represent an average of ~13.5% (mole basis) of total sugars, indicating the presence of glucose at ~86.5% (mole basis). Initial glucose concentrations, measured at time zero (T_0) of batch fermentation, ranged from 1.27±0.06 to 0.83±0.04 g/g-starch and are expressed in Table 4.5, along with maltose, maltotriose and DP4 concentrations. It is noted that CDC Falcon appears to have a markedly lower initial glucose concentration (0.83±0.04) than other samples. Due to discrepancy in sample time for T_0, ranging from 5-15 min depending on

the order of sample preparation, it is possible that AMG had not had sufficient opportunity to hydrolyze the CDC Falcon wort, as evidenced in the elevated DP4 and maltose concentrations. CDC Falcon, in fact, had ~30% (mole basis) of its total sugars derived from larger polysaccharides, significantly higher than the average of ~13.5%. Additionally, it is noted that the theoretical conversion limit of glucose to starch is quoted as 1.11 g-glucose/g-starch (Wu et al. 2007). As previously mentioned, background sugars imparted from enzyme preparations could not be removed from experimental flasks and are likely elevating observed values above theoretical limits.

Table 5.5. Fermentable sugar concentration at zero (T_0) and 24 (T_{24}) hours after SSF for various substrates.

		DP4 (g/g-starch)		Maltotriose (g/g-starch)		Maltose (g/g-starch)		Glucose (g/g-starch)	
Class	Variety	T_{0h}	T_{24h}	T_{0h}	T_{24h}	T_{0h}	T_{24h}	T_{0h}	T_{24h}
CPSR	S700 PR	0.08 ± 0.01	0.01 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	1.02 ± 0.03	0.02 ± 0.01
CWHWS	Snowbird	0.07 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.93 ± 0.02	0.01 ± 0.01
CWSWS	AC Andrew	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.01 ± 0.04	0.03 ± 0.01
CWRW	CDC Falcon	0.20 ± 0.06	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.01	0.16 ± 0.02	0.00 ± 0.00	0.83 ± 0.04	0.03 ± 0.02
	Buteo	0.07 ± 0.09	0.00 ± 0.00	0.04 ± 0.00	0.01 ± 0.01	0.07 ± 0.06	0.01 ± 0.02	1.27 ± 0.06	0.03 ± 0.00
Triticale	Banjo	0.04 ± 0.02	0.00 ± 0.00	0.05 ± 0.00	0.01 ± 0.00	0.01 ± 0.05	0.00 ± 0.02	1.05 ± 0.07	0.03 ± 0.01
CMC	Corn	0.07 ± 0.04	0.00 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.03 ± 0.03	0.02 ± 0.01	1.14 ± 0.06	0.03 ± 0.01

* Statistical analysis was not performed on results

* Reported values are averages of all replicates (n=3) and their standard deviations

The efficiency of sugar utilization during fermentation is observed by assessing the quantity of available sugars consumed during the fermentation period. The sugars remaining, referred to as residual sugar, are considered to be either inaccessible to AMG, due to the presence of α-(1,6)-glycosidic branch points preventing entry of substrate into the enzyme, as in the case with isomaltose or panose, or to be inaccessible to yeast for consumption. Residual sugar compositions, described in Table 5.5, are similar between

samples. Total extinction of both DP4 and maltose is observed for all samples during SSF. Maltotriose, however, appears to have residual content averaging ~0.03g/g-starch across all substrates. Incomplete utilization of wort maltotriose is described as 'a common problem encountered by a number of breweries'(Zheng et al. 1994), and is consistent with findings presented here. Residual glucose concentration ranges from 0.03-0.01g/g-starch and represents the most prevalent sugar type in the post-fermentation liquor. The bioavailability of sugars, described in Equation 5.1, was calculated and compared for each sample. When considering the large standard deviations present, bioavailability appears similar between substrates, ranging from 94.58±8.83% to 97.54±2.68%. According to these results, the sugars present in the post-saccharification broth appear to be equivalently available for consumption by yeast, independent of substrate. The action of AMG on liquefied wort, however, appears to generate disproportionately large amounts of glucose in both CMC and Buteo, relative to the other substrates.

Table. 5.6. Starch bioavailability for various substrates during SSF with *S.cerevisiae.*

Class	Variety	Starch Bioavailability (%)
CPSR	S700 PR	95.10 ± 3.92
CWHWS	Snowbird	97.54 ± 2.68
CWSWS	AC Andrew	95.30 ± 5.39
CWRW	CDC Falcon	94.67 ± 9.44
	Buteo	96.78 ± 12.21
Triticale	Banjo	96.29 ± 11.25
CMC	Corn	94.58 ± 8.83

*values calculated according to Eqn. 5.11

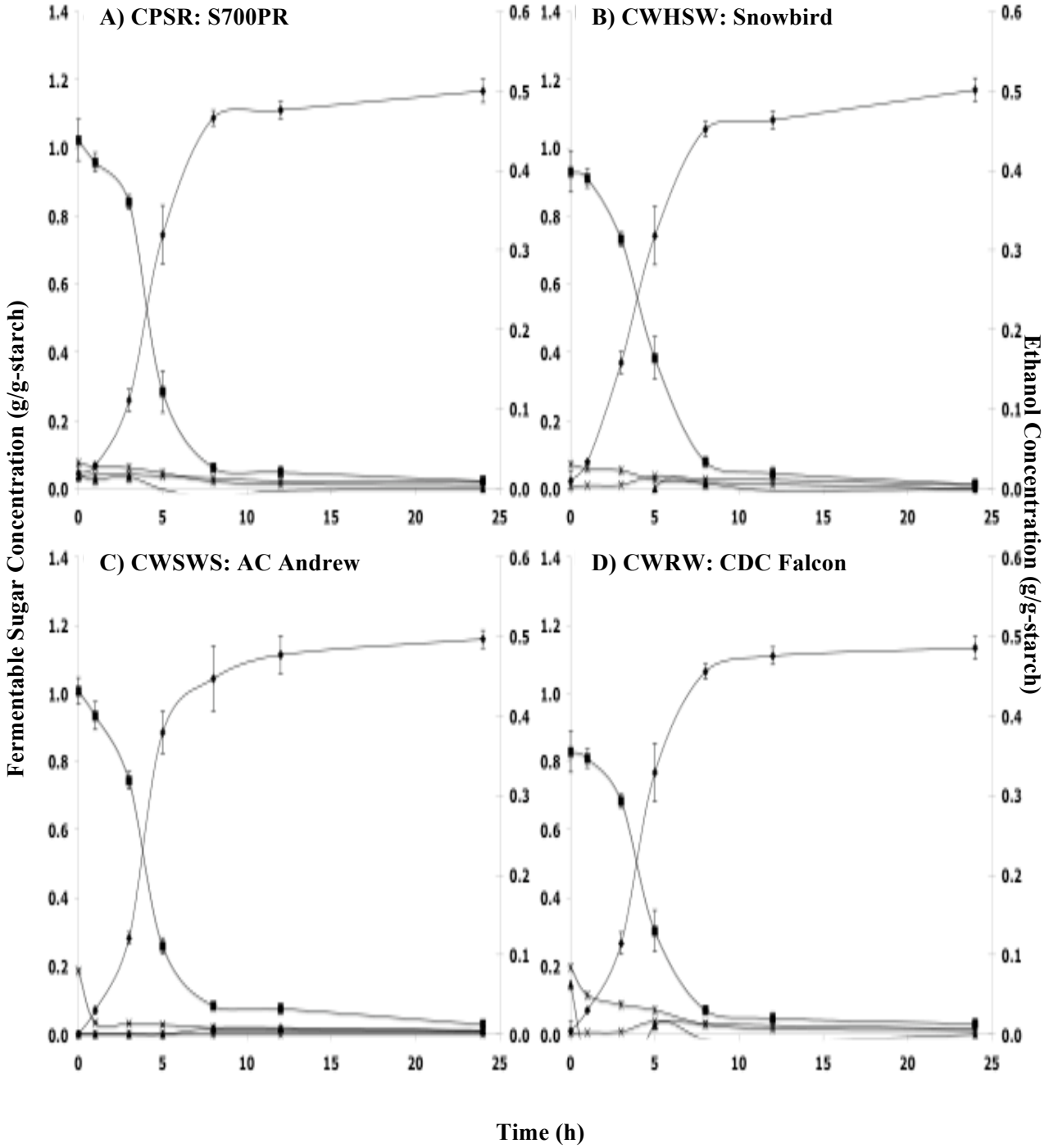

Figure 5.7. Time course of glucose, maltose, maltotriose and DP4 concentration for various starch substrates during SSF with concomitant ethanol production. Symbols: ◆, Ethanol; ■, Glucose; Δ, Maltose; ✕, Maltotriose; ●, DP4. Error bars represent the standard deviation for replicates (n=3).

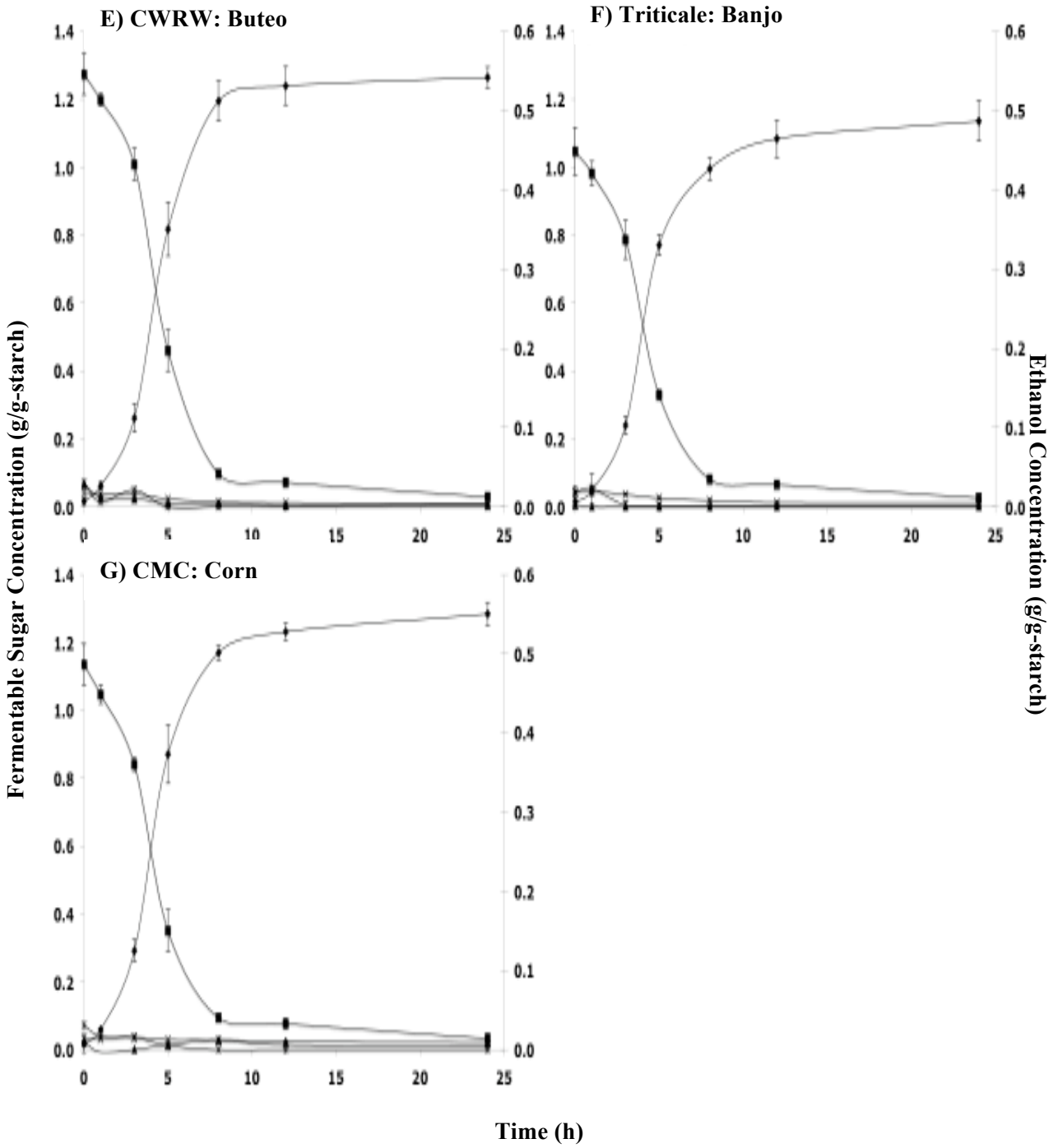

Figure 5.7. (cont) Time course of glucose, maltose, maltotriose and DP4 concentration for various starch substrates during SSF with concomitant ethanol production. Symbols: ◆, Ethanol; ■, Glucose; Δ, Maltose; ✕, Maltotriose; ●, DP4. Error bars represent the standard deviation for replicates (n=3).

5.4 Conclusions

Liquefaction performance was found to be similar between samples, indicating no major differences in the quantities of maltose, maltotriose, maltotetrose or larger dextran concentrations in the respective worts. During saccharification and fermentation, however, striking differences in glucose generation were observed, averaging 1.21 g/g-starch (at T_0) for the high glucose cohort, compared to a range of 0.83 to 1.05 g/g-starch for the low yielding cohort. In general, the pattern of sugar generation appears to be consistent with ethanol and biomass production. Sugar utilization appears consistent between samples, ranging from 95-97%, and unrelated to observed differences in end-product production. CMC and Buteo, observed to have the highest ethanol and biomass yields, were also observed to have the highest concentrations of glucose at T_0, whereas the low glucose cohort exhibited depressed production of ethanol and biomass. It appears high glucose concentration in the fermentation broth did not correlate to differences in kinetic parameters of *S.cerevisiae* growth, but rather imparted ample nutrients for yeast cells to remain in near exponential growth for an extended period. The close relationship between exponential growth and ethanol production, as previously mentioned, suggests this additional growth generated the elevated ethanol yields observed in CMC and Buteo.

Chapter 6: Conclusions

6.1 Wheat Starch Ideal for Bioethanol End-use

Presently no evaluative criteria, other than cost and availability, are employed in the selection of grains destined for use as bioethanol feedstock. In an effort to establish guidelines for wheat selection and breeding, this study attempts to elucidate the relationship between physicochemical parameters of starch and susceptibility to enzymatic hydrolysis and concomitant fermentation performance by *S.cerevisiae*. The identification of starch properties yielding high conversion efficiencies of starch to fermentable sugars is a critical step in the generation of wheat cultivars tailored to the needs of bioethanol producers. Table 6.1 describes correlation between select physicochemical parameters of starch and observed fermentative ethanol yields, discussed in chapter 4 and 5 of this report, respectively. The physicochemical parameters of starch that appear to correlate with ethanol yield, and therefore could possibly act as predictive characteristics of starches amenable to bioethanol application, include amylose to amylopectin ratio and reducing sugar yield. Less significant correlation between selected physicochemical parameters and ethanol yield were observed for peak temperature, a thermal property related to crystallinity and gelatinization performance, and breakdown and trough viscosity, pasting properties related to starch solubility.

Table 6.1. Linear correlation between select physicochemical parameters of starch and fermentative ethanol yield with *S.cerevisiae* during SSF.

Physicochemical Parameter	r	P-value
Amylose/Amylopectin Content		
Amylose Content (%)	-0.784	<0.05
Thermal Properties		
T_p (^{0}C)	0.680	ns
T_o (^{0}C)	0.297	ns
T_c (^{0}C)	0.541	ns
Particle Size Distribution		
A-Type Granule (>10μm)	0.374	ns
Amylopectin Fine Structure		
DP 6-12 Fraction	0.231	ns
Pasting Properties		
Peak Viscosity (cP)	0.328	ns
Breakdown Viscosity (cP)	0.629	ns
Final Viscosity (cP)	0.487	ns
Reducing Sugar Content		
Yield (mg-glucose equivs/g-starch)	-0.921	<0.01

* ns = not significant at P<0.05

A reduction in amylose content has been positively correlated with amylolyitc digestibility under gelatinized conditions (Liu et al. 2007; Matser and Steeneken 1998) and has been found to yield higher ethanol conversion efficiencies than higher amylose counterparts (Wu et al. 2006, Wu et al. 2007, Zhao et al. 2009). Confirming the findings of Wu et al. (2006), Wu et al. (2007) and Zhao et al. (2009), amylose content is observed in this study to have a moderate negative correlation with ethanol yield (r=-0.784, P<0.05), suggesting even subtle decreases in wheat starch amylose content (~4%) correspond to increases in generated ethanol. Recent findings suggest high amylose starches appear to have numerous drawbacks in regards to ease of gelatinization and resultant performance under the conditions of liquefaction. Wu et al. (2007) suggests that

as amylose content increases the quantity of resistant starch, those oligosaccharides inaccessible to amylolytic enzymes, also increases. Fermentation is likely inhibited, then, not only by a reduction in total available sugars but also by the higher viscosity mashes created when liquefaction is incomplete. High amylopectin starches, therefore, are being explored with great enthusiasm and Lacerenza et al. (2008) has already recommended waxy wheat for application as bioethanol feedstock, despite commercial development being years away.

Reducing sugar yield, a rough measure of oligosaccharide chain length distribution, shows a strong negative correlation ($r=-0.921$, $P<0.01$) with ethanol yield. Low quantities of reducing sugars present in the enzyme hydrolyzate can be interpreted as an increased presence of longer chain oligosaccharides, resulting in fewer exposed reducing ends. The action of AMG on liquefied wort possessing high quantities of longer chain oligosaccharides appears to generate greater quantities of glucose, as discussed in section 4.3.4. The exact mechanism of action of AMG on long as compared to short chain oligosaccharides is unclear. However, it is reported that AMG preferentially attacks 'longer glucose polymers' (Montesinos and Navarro 2000b), perhaps explaining the increased efficacy, in regards to glucose generation, observed here. Of note is the relationship between reducing sugar yield and amylose content, which is observed to positively correlate ($r=0.577$, $P<0.05$), as discussed in section 4.3.8 of this thesis. It appears that starch with comparatively low amylose content generates a pattern of α–amylolytic hydrolysis consistent with maximum ethanol yield, likely via the production of increased quantities of longer chain oligosaccharides.

Additionally, peak temperature is observed to have moderate positive correlation with ethanol yield ($r=0.68$, not significant at $P<0.05$), suggesting higher peak

temperatures of starch may generate favorable liquefaction conditions. The small sample size included here (n=7), however, makes interpretation difficult, as resultant findings are statistically insignificant. Peak temperature is reportedly influenced by amylose content (Gupta et al. 2009; Hoseney 1998; Tester and Morrison 1990) and high amylopectin starches are reported to require higher energies of gelatinization due to increased crystallinity (Hung et al. 2006; Noda et al. 2002; Van Hung et al. 2007). Starches included in this study showed no meaningful correlation between amylose content and thermal properties, likely due to the narrow range of amylose content observed amongst included samples. Somewhat confounding to these results, the study also suggests high peak temperature, presumably due to increased amylopectin/crystallinity, appears to trend with increased fermentation performance, a finding consistent with the current accepted paradigm for the relationship between amylopectin and enzymatic digestibility. Offering some explanation to observed differences in enzymatic digestibility between high and low amylose content cultivars, several authors have suggested high amylose content starches are not as easily gelatinized as high amylopectin counterparts (Wu et al. 2007; Zhao et al. 2009). In fact, Wu et al. (2007) claims cooking high-amylose starches at temperatures above 120^0C still does not yield conversion efficiencies equal to normal or low amylose starch cooked at ~70^0C, the temperature required to achieve complete granular disruption/dissolution for waxy wheat starch. The observed relationship between ethanol yield and peak temperature, when viewed in light of the established relationship between thermal properties and amylopectin content, is perhaps appropriately interpreted as amylose content bearing concurrent influence on ethanol production and observed thermal properties, rather than thermal properties directly influencing ethanol production.

Additional correlation between ethanol yield and breakdown viscosity was observed for study samples, ($r=0.629$, not significant at $P<0.05$). As for peak temperature, the small sample size included here ($n=7$) makes interpretation difficult for these findings, as results are statistically insignificant. Despite the lack of statistical significance the relationship between ethanol yield and breakdown viscosity is consistent with the findings of Wu et al. (2007) and Zhao et al. (2009). Study samples exhibiting increased solubility did, in general, exhibit higher ethanol yields. As with peak temperature, the relationship of amylose content to breakdown viscosity likely underlies the purported relationship of starch solubility to ethanol performance. Several authors have reported that amylose content is indeed influential to pasting parameters (Abdel-Aal et al. 2002; Gupta et al. 2009; Zeng et al. 1997) and, in accord with these findings, breakdown viscosity is observed in this study to have negative correlation with amylose content ($r=-0.363$, not significant at $P<0.05$). In general, low amylose starch exhibits increased solubility as compared to high amylose counterparts, a property highly amenable to enzymatic digestibility. Zhao et al. (2009) states that 'for waxy wheat cultivars, gelatinized starch granules were more susceptible to breakdown under liquefaction conditions; thus, starch molecules were more extensively exposed and more accessible to heat-stable α-amylase.'

Critical to starch conversion efficiency into fermentable sugars is the granular dissolution achieved during gelatinization and the availability of solubilized material to liquefying enzymes. Therefore, a salient feature in the generation of cultivars tailored to the needs of the bioethanol industry include physicochemical parameters of starch lending themselves to high conversion efficiency under the conditions of gelatinization and liquefaction. The relationship of amylose/amylopectin content to reducing sugar yield, thermal and pasting properties suggests this one parameter may serve as the best

predictive metric for anticipating liquefaction and fermentation performance from select wheat starches. However, due to the small sample size included here, future work is recommended to verify study findings. The encompassing recommendation of this work is the selection of wheat starch with the highest amylopectin content achievable, theoretically delivering starch optimized for both rapid and complete degradation by industrial enzymes.

6.2 Limitations and Future Work

Despite the suggested relationship between amylose/amylopectin ratio to both thermal and pasting properties and the potential to use only the former as a predictive metric for ethanol generation, the value of the later measurements to assess wheat performance during whole grain industrial processing should not be discounted. Mash viscosity, a parameter closely tied to properties of gelatinization and solubility, was not a factor in this study due to the low concentration system employed: ~1% (w/v) pure starch feedstock. Industrial conditions, conversely, employ upwards of 20% (w/v) whole grain feedstock which exhibits high viscosity during mashing and often suffers mass transfer issues as a result. Due to limitations in study design, extrapolating study findings to whole grain systems at high solid loading is difficult. Future work is recommended to assess the impact of whole grain processing on ethanol generation, and, in particular, the behavior of starch within that system.

6.3 Western Canadian Cultivars Recommended for Bioethanol End-Use

Whole kernel characteristics previously reported as ideal for bioethanol end-use include low protein, high starch, and large kernel size. Table 6.2 summarizes the key whole kernel composition and starch physicochemical findings of those cultivars included in fermentation trials, along with estimated ethanol production per tonne of whole grain.

Those cultivars exhibiting high starch/low protein include AC Andrew, CDC Falcon, and Buteo. AC Andrew, however, had a starch content ~4% higher than either CDC Falcon or Buteo, and comparable to CMC. According to section 4.1.1 of this thesis seed size was shown to correlate with starch content ($r=0.56$, $P<0.05$), suggesting high seed weight may be an indicator of high starch load in the kernel. Of wheat cultivars measured, AC Andrew exhibited both the highest seed weight and starch content. Despite high starch content and seed weight, AC Andrew exhibited the highest quantity of amylose, a feature possibly detrimental to conversion efficiency. Buteo and Snowbird, conversely, exhibited the lowest quantities of amylose, indicating their starches may be the most susceptible to enzymatic hydrolysis.

Table 6.2. Whole kernel chemical composition, thousand kernel weight (1000KWT), amylose content, and calculated ethanol yield per tonne whole grain for select cultivars.

Class	Variety	Protein (%, dwb)	Total Starch (%, dwb)	Amylose (%)	1000KWT (g, dwb)	Ethanol Yield* (L/tonne)
CPSR	S700 PR	14.9	60.61 ± 3.01	28.17 ± 0.28	35.59	384.06
CWHWS	Snowbird	14.9	60.93 ± 0.07	26.40 ± 0.69	38.04	387.30
CWSWS	AC Andrew	10.1	68.60 ± 0.09	30.16 ± 1.39	38.62	431.82
CWRW	CDC Falcon	10.7	63.81 ± 0.26	28.33 ± 1.04	34.99	392.87
	Buteo	11.3	64.20 ± 0.57	27.16 ± 0.53	32.80	441.10
Triticale	Banjo	13.6	62.26 ± 2.90	28.97 ± 1.36	45.90	384.32
CMC	Corn	9.9	71.55 ± 0.24	25.49 ± 0.53	325**	498.92

* Ethanol yield (L/tonne) calculated based on experimental yield data expressed in Table 5.3 as $Y_{E/S}$, 1000KWT and starch content findings. A sample calculation is available in Appendix A.
** Literature value (Hui 2006).

Experimental findings for pure starch fermentation, detailed in Section 4.3.2 of this thesis, indicates Buteo had comparable fermentation performance to CMC, and yielded roughly 10% more ethanol than other cultivars of wheat. However, if observed

ethanol conversion efficiencies on pure starch are used to estimate possible whole grain conversion, which considers total kernel starch content and 1000KWT, AC Andrew and Buteo, interestingly, have similar whole grain conversion efficiencies (Table 6.2). Ethanol yield for whole kernel conversion of S700PR, Snowbird, CDC Falcon and Banjo ranges from ~384 to 392 L/tonne, whereas AC Andrew and Buteo range from ~432 to 441 L/tonne. For the case of AC Andrew, lower ethanol yield on pure starch was mitigated by higher total starch content. Buteo, conversely, with a higher ethanol yield on pure starch than AC Andrew, theoretically produces marginally more ethanol on whole grain than AC Andrew, despite possessing ~4% lower starch content. CMC with a starch content roughly 10% higher than wheat cultivars included in this study, and the highest measured ethanol yield on pure starch, theoretically delivers ~500L/tonne of ethanol when considering whole grain processing. In general, amongst wheat, AC Andrew and Buteo appear to be equally well suited to whole grain bioethanol processing and should likely be given equal consideration as potential feedstock for that industry.

References

Abd-Aziz, S., Ang, D.C., Yusof, H.M., Karim, M.I.A., Ariff, A.B., Uchiyama, K., and Shioya, S. 2001. Effect of C/N ratio and starch concentration on ethanol production from sago starch using recombinant yeast. World Journal of Microbiology & Biotechnology **17**(7): 713-719.

Abdel-Aal, E.S.M., Hucl, P., Chibbar, R.N., Han, H.L., and Demeke, T. 2002. Physicochemical and structural characteristics of flours and starches from waxy and nonwaxy wheats. Cereal Chemistry **79**(3): 458-464.

Agarwal, A.K. 2007. Biofuels (alcohols and biodiesel) applications as fuels for internal combustion engines. Progress in Energy and Combustion Science **33**(3): 233-271.

Agu, R.C., Bringhurst, T.A., and Brosnan, J.M. 2006. Production of grain whisky and ethanol from wheat, maize and other cereals. Journal of the Institute of Brewing **112**(4): 314-323.

American Association of Cereal Chemists International, *Approved Methods of the AACC*, 10th edition. AACC, St Paul, MN (2000)

Andersson, R., Westerlund, E., Tilly, A.C., and Aman, P. 1993. Natural Variations in the Chemical-Composition of White Flour. Journal of Cereal Science **17**(2): 183-189.

Antizar-Ladislao, B., and Turrion-Gomez, J.L. 2008. Second-generation biofuels and local bioenergy systems. Biofuels Bioproducts & Biorefining-Biofpr **2**(5): 455-469.

Ao, Z.H., and Jane, J.L. 2007. Characterization and modeling of the A- and B-granule starches of wheat, triticale, and barley. Carbohydrate Polymers **67**(1): 46-55.

Ao, Z.H., Simsek, S., Zhang, G.Y., Venkatachalam, M., Reuhs, B.L., and Hamaker, B.R. 2007. Starch with a slow digestion property produced by altering its chain length, branch density, and crystalline structure. Journal of Agricultural and Food Chemistry **55**(11): 4540-4547.

Bai, F.W., Anderson, W.A., and Moo-Young, M. 2008. Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnology Advances **26**(1): 89-105.

Barron, C., Surget, A., and Rouau, X. 2007. Relative amounts of tissues in mature wheat (Triticum aestivum L.) grain and their carbohydrate and phenolic acid composition. Journal of Cereal Science **45**(1): 88-96.

Bechtel, D.B., Zayas, I., Kaleikau, L., and Pomeranz, Y. 1990. Size-distribution of wheat-starch granules during endopserm development. Cereal Chemistry **67**(1): 59-63.

Bertilsson, M., Olofsson, K., and Liden, G. 2009. Prefermentation improves xylose utilization in simultaneous saccharification and co-fermentation of pretreated spruce. Biotechnology for Biofuels **2**(8): 1754-6834.

Bettiga, M., Hahn-Hagerdal, B., and Gorwa-Grauslund, M.F. 2008. Comparing the xylose reductase/xylitol dehydrogenase and xylose isomerase pathways in arabinose and xylose fermenting Saccharomyces cerevisiae strains. Biotechnology for Biofuels **1**(16): 1754-6834.

Birol, G., Onsan, Z.I., Kirdar, B., and Oliver, S.G. 1998. Ethanol production and fermentation characteristics of recombinant Saccharomyces cerevisiae strains grown on starch. Enzyme and Microbial Technology **22**(8): 672-677.

Boltz, K.W., and Thompson, D.B. 1999. Initial heating temperature and native lipid affects ordering of amylose during cooling of high-amylose starches. Cereal Chemistry **76**(2): 204-212.

Brown, L.R. 2008. Why Ethanol Production Will Drive Food Prices Even Higher in 2008. Earth Policy Institute, http://www.earth-policy.org/index.php?/plan_b_updates/2008/update69.

Cardona, C.A., and Sanchez, O.J. 2007. Fuel ethanol production: Process design trends and integration opportunities. Bioresource Technology **98**(12): 2415-2457.

CGC. 2009a. Canadian wheat. Canadian Grain Commission, http://www.grainscanada.gc.ca/research-recherche/dexter/cw-bc/cw-bc-5-eng.htm#f.

CGC. 2009b. Canadian wheat classes. Canadian Grain Commission, http://www.grainscanada.gc.ca/wheat-ble/classes/classes-eng.htm.

CGC. 2010. Wheat methods and tests used to measure quality. Canadaian Grain Commission, http://www.grainscanada.gc.ca/wheat-ble/method-methode/wmtm-mmab-eng.htm

Charles, M.B., Ryan, R., Ryan, N., and Oloruntoba, R. 2007. Public policy and biofuels: The way forward? Energy Policy **35**(11): 5737-5746.

Chung, J.H., Han, J.A., Yoo, B., Seib, P.A., and Lim, S.T. 2008. Effects of molecular size and chain profile of waxy cereal amylopectins on paste rheology during retrogradation. Carbohydrate Polymers **71**(3): 365-371.

CRFA. 2009. Canadian Renwable Fuels Association, http://www.greenfuels.org/lists.php.

CWB. 2009. Quality of Western Canadian Wheat Canadian Wheat Board, http://www.grainscanada.gc.ca/wheat-ble/harvest-recolte/2009/hqw09-qrb09-eng.htm.

Dale, B. 2008. Biofuels: Thinking clearly about the issues. Journal of Agricultural and Food Chemistry **56**(11): 3885-3891.

Dale, R.T., and Tyners, W.E. 2006. Purdue University Working Papers; Economic and Technical Analysis of Ethanol Dry Milling: Model Description. Purdue University College of Agriculture, Department of Agricultural Economics, http://ideas.repec.org/p/pae/wpaper/06-04.html

Das Neves, M.A. 2006. Bioethanol production from wheat milling by-products. *In* Appropriate Technology and Sciences for Sustainable Development. Universtiy of Tsukuba, Ibaraki, Japan.

Das Neves, M.A., Kimura, T., Shimizu, N., and Shiiba, K. 2006. Production of alcohol by simultaneous saccharification and fermentation of low-grade wheat flour. Brazilian Archives of Biology and Technology **49**(3): 481-490.

Das Neves, M.A., Shimizu, N., Kimura, T., and Shiiba, K. 2007. Kinetics of bioethanol production from wheat milling by-products. Journal of Food Process Engineering **30**(3): 338-356.

Dengate, H., and Meredith, P. 1984. Variation in size disbribution of starch granules from wheat-grain. Journal of Cereal Science **2**(2): 83-90.

Diouf, J. 2007. Biofuels a disaster for world food. European Union Coherence, October 31.

Dreher, M.L., Dreher, C.J., and Berry, J.W. 1984. Starch digestibilty of foods - A nutrional perspective. Crc Critical Reviews in Food Science and Nutrition **20**(1): 47-71.

Dronzek, B.L., Bushuk, W., and Hwang, P. 1972. Scanning Electron-Microscopy of Starch from Sprouted Wheat. Cereal Chemistry **49**(2): 232-&.

Du, X., and Hayes, D.J. 2008. Working Paper 08-WP 467; The Impact of Ethanol Production on U.S. and Regional Gasoline Prices and on the Profitability of the U.S. Oil Refinery Industry. Center for Agricultural and Rural Development, Iowa State University, http://www.card.iastate.edu/publications/synopsis.aspx?id=1076.

Eglinton, J.M., Heinrich, A.J., Pollnitz, A.P., Langridge, P., Henschke, P.A., and Lopes, M.D. 2002. Decreasing acetic acid accumulation by a glycerol overproducing strain of Saccharomyces cerevisiae by deleting the ALD6 aldehyde dehydrogenase gene. Yeast **19**(4): 295-301. doi:10.1002/yea.834.

EIA. 2009a. Imports/Exports & Movements. Energy Information Administration, Offical Energy Statistics from the U.S. Government, http://tonto.eia.doe.gov/dnav/pet/pet_move_wkly_dc_NUS-Z00_mbblpd_w.htm.

EIA. 2009b. Petroleum Consumption/Sales. Energy Information Administration: Official Energy Statistics from the U.S. Government, http://tonto.eia.doe.gov/dnav/pet/pet_cons_psup_dc_nus_mbblpd_a.htm.

EIA. 2009c. Weekly U.S. All Grades All Formulations Retail Gasoline Prices. Energy Information Administration, Official Energy Statistics from the U.S. Government, http://tonto.eia.doe.gov/dnav/pet/hist/LeafHandler.ashx?n=PET&s=MG_TT_US&f=W.

Ernandes, J.R., Williams, J.W., Russell, I., and Stewart, G.G. 1993. Effect of yeast adaptation to maltose utilization on sugar uptake during the fermentation of brewers wort. Journal of the Institute of Brewing **99**(1): 67-71.

Farrell, A.E. 2006. Ethanol can contribute to energy and environmental goals Science **312**(5781): 1748-1748.

Fink, M., Feller, C., Scharpf, H.C., Weier, U., Maync, A., Ziegler, J., Paschold, P.J., and Strohmeyer, K. 1999. Nitrogen, phosphorus, potassium and magnesium contents of field vegetables - Recent data for fertiliser recommendations and nutrient balances. Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde **162**(1): 71-73.

Franco, C.M.L., Wong, K.S., Yoo, S.H., and Jane, J.L. 2002. Structural and functional characteristics of selected soft wheat starches. Cereal Chemistry **79**(2): 243-248.

Fuel Ethanol. 2009. Ethanol Market Weekly News and Market Report. Ethanol Market http://www.ethanolmarket.com/fuelethanol.html.

Fujii, N., Oki, T., Sakurai, A., Suye, S., and Sakakibara, M. 2001. Ethanol production from starch by immobilized Aspergillus awamori and Saccharomyces pastorianus using cellulose carriers. Journal of Industrial Microbiology & Biotechnology **27**(1): 52-57.

Gong, C.S., Ladisch, M.R., and Tsao, G.T. 1981. Production of ethanol from wood hemicellulose hydrolyzates by xylose-fermenting yeast mutant, Candida-sp XF-217. Biotechnology Letters **3**(11): 657-662.

Govindaswamy, S., and Vane, L.M. 2010. Multi-stage continuous culture fermentation of glucose-xylose mixtures to fuel ethanol using genetically engineered Saccharomyces cerevisiae 424A. Bioresour Technol **101**(4): 1277-1284.

Graybosch, R.A. 1998. Waxy wheats: Origin, properties, and prospects. Trends in Food Science & Technology **9**(4): 135-142.

Gupta, M., Bawa, A.S., and Semwal, A.D. 2009. Morphological, Thermal, Pasting, and Rheological Properties of Barley Starch and Their Blends. International Journal of Food Properties **12**(3): 587-604.

Guraya, H.S., Kadan, R.S., and Champagne, E.T. 1997. Effect of rice starch-lipid complexes on in vitro digestibility, complexing index, and viscosity. Cereal Chemistry **74**(5): 561-565.

Hahn, R.W. 2008. Reg-Marktes Center Working Paper No. 08-02; Ethanol: Law, Economics, and Politics, http://ssm.com/abstract=1082079.

Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., and Gorwa-Grauslund, M.F. 2007. Towards industrial pentose-fermenting yeast strains. Applied Microbiology and Biotechnology **74**(5): 937-953.

Hanashiro, I., Abe, J., and Hizukuri, S. 1996. A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. Carbohydrate Research **283**: 151-159.

Hansen, J., Sato, M., Ruedy, R., Lo, K., Lea, D.W., and Medina-Elizade, M. 2006. Global temperature change. Proceedings of the National Academy of Sciences of the United States of America **103**(39): 14288-14293.

Hashimoto, S., Shogren, M.D., and Pomeranz, Y. 1987. Cereal Pentosans - Their Estimation and Significance .1. Pentosans in Wheat and Milled Wheat Products. Cereal Chemistry **64**(1): 30-34.

Hebeda, R.E., and Leach, H.W. 1974. Nature of insoluble starch particles in liquefied corn-starch hydrolysates Cereal Chemistry **51**(2): 272-281.

Hill, J., Nelson, E., Tilman, D., Polasky, S., and Tiffany, D. 2006. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proceedings of the National Academy of Sciences of the United States of America **103**(30): 11206-11210.

Hizukuri, S. 1986. Polymodal distribution of the chain lengths of amylopectins, and its significance. Carbohydrate Research **147**(2): 342-347.

Hizukuri, S., Abe, J., and Hanashiro, I. 1996. Starch: Analytical Aspects. *In* Carbohydrates in Food. *Edited by* Ann-Charlotte Eliasson. Taylor & Francis Group, Boca Raton, FL.

Holm, J., Lundquist, I., Bjorck, I., Eliasson, A.C., and Asp, N.G. 1988. Degree of Starch Gelatinization, Digestion Rate of Starch Invitro, and Metabolic Response in Rats. American Journal of Clinical Nutrition **47**(6): 1010-1016.

Hoogwijk, M., Faaij, A., Eickhout, B., de Vries, B., and Turkenburg, W. 2005. Potential of biomass energy out to 2100, for four IPCCSRES land-use scenarios. Biomass & Bioenergy **29**(4): 225-257.

Hoseney, R.C. (ed). 1998. Principles of Cereal Science and Technology. American Association of Cereal Chemists, St. Paul, MN.

Hruskova, M., and Svec, I. 2009. Wheat Hardness in Relation to Other Quality Factors. Czech Journal of Food Sciences **27**(4): 240-248.

Huang, J.R., Schols, H.A., van Soest, J.J.G., Jin, Z.Y., Sulmann, E., and Voragen, A.G.J. 2007. Physicochemical properties and amylopectin chain profiles of cowpea, chickpea and yellow pea starches. Food Chemistry **101**(4): 1338-1345.

Hui, Y. 2006. Cereals biology, pre- and post- harvest management. *In* Handbook of Food Scicne, Technology & Engineering. Vol. 1. *Edited by* Y. Hui. Taylor & Francis Group, Boca Raton, FL.

Hung, P.V., Maeda, T., and Morita, N. 2006. Waxy and high-amylose wheat starches and flours - characteristics, functionality and application. Trends in Food Science & Technology **17**(8): 448-456.

IEA. 2005. International Energy Agency - Resources to Reserves: Oil & Gas Technologies for the Energy Markets of the Future. International Energy Agency, Head of Publications Service, http://www.iea.org/publications/free_new_Desc.asp?PUBS_ID=1568.

Ingledew, W.M. (ed). 1999. Alcohol Production by *Saccharomyces cerevisiae*: a yeast primer. University Press, Nottingham, UK.

Izydorczyk, M., Biliaderis, C.G., and Bushuk, W. 1991. Comparison of the Structure and Composition of Water-Soluble Pentosans from Different Wheat-Varieties. Cereal Chemistry **68**(2): 139-144.

Jane, J., Chen, Y.Y., Lee, L.F., McPherson, A.E., Wong, K.S., Radosavljevic, M., and Kasemsuwan, T. 1999. Effects of amylopectin branch chain length and amylose content on the gelatinization and pasting properties of starch. Cereal Chemistry **76**(5): 629-637.

Jenkins, P.J., and Donald, A.M. 1995. The influence of amylose on starch granule structure. *In Conference Proceedings: 4th International Workshop on Oligo- and Polysacchardies*. International Journal of Biological Marcomolecules **17**(6): 315-321.

Jenkins, P.J., and Donald, A.M. 1998. Geratinisation of starch: a combined SAXS/WAXS/DSC and SANS study. Carbohydrate Research **308**(1-2): 133-147.

Kappeli, O. 1986. Regulation of carbon metabolism in Saccharomyces cerevisiae and related yeasts Advances in Microbial Physiology **28**: 181-209.

Kim, S., and Dale, B.E. 2004. Global potential bioethanol production from wasted crops and crop residues. Biomass & Bioenergy **26**(4): 361-375.

Kindred, D.R., Verhoeven, T.M.O., Weightman, R.M., Swanston, J.S., Aguc, R.C., Brosnan, J.M., and Sylvester-Bradley, R. 2008. Effects of variety and fertiliser nitrogen on alcohol yield, grain yield, starch and protein content, and protein composition of winter wheat. Journal of Cereal Science **48**(1): 46-57.

Kleijer, G., Levy, L., Schwaerzel, R., Fossati, D., and Brabant, C. Relationship between test weight and several quality parameters in wheat. Agrarforschung **14**(11-12): 548-555

Koplow, D. 2006. The Global Studies Initiative (GSI): Biofuels - At What Cost? Government Support for Ethanol and Biodiesel in the United States. The International

Institute for Sustainable Development (IISD), http://www.globalsubsidies.org/IMG/pdf/biofuels_subsidies_us.pdf.

Koutinas, A.A., Wang, R., and Webb, C. 2004. Evaluation of wheat as generic feedstock for chemical production. Industrial Crops and Products **20**(1): 75-88.

Krisch, J., and Szajani, B. 1997. Ethanol and acetic acid tolerance in free and immobilized cells of Saccharomyces cerevisiae and Acetobacter aceti. Biotechnology Letters **19**(6): 525-528.

Krueger, B.R., Knutson, C.A., Inglett, G.E., and Walker, C.E. 1987. A differential scanning calorimetry study on the effect of annealing on gelatinization behaviour of corn starch Journal of Food Science **52**(3): 715-718.

Kulp, K. 1973. Characteristics of Small-Granule Starch of Flour and Wheat. Cereal Chemistry **50**(6): 666-679.

Kwasniewska-Karolak, I., Nebesny, E., and Rosicka-Kaczmarek, J. 2008. Characterization of amylose-lipid complexes derived from different wheat varieties and their susceptibility to enzymatic hydrolysis. Food Science and Technology International **14**(1): 29-37.

Lacerenza, J.A., Martin, J.M., Talbert, L.E., Lanning, S.P., and Giroux, M.J. 2008. Relationship of ethanol yield to agronomic and seed quality characteristics of small grains (vol 85, pg 322, 2008). Cereal Chemistry **85**(4): 578-578.

Lackner, K.S., and Sachs, J.D. 2005. A robust strategy for sustainable energy. Brookings Papers on Economic Activity **13**(2): 215-284.

Lee, M.R., Swanson, B.G., and Baik, B.K. 2001. Influence of amylose content on properties of wheat starch and breadmaking quality of starch and gluten blends. Cereal Chemistry **78**(6): 701-706.

Lee, Y.S., Lee, W.G., Chang, Y.K., and Chang, H.N. 1995. Modeling of ethanol-production by Saccharomyces cerevisiae from a glucose and maltose mixture Biotechnology Letters **17**(8): 791-796.

Lin, P.Y., and Czuchajowska, Z. 1997. Starch properties and stability of club and soft white winter wheats from the Pacific Northwest of the United States. Cereal Chemistry **74**(5): 639-646.

Lindeboom, N., Chang, P.R., and Tyler, R.T. 2004. Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches: A review. Starch-Starke **56**(3-4): 89-99.

Liu, Q., Gu, Z., Donner, E., Tetlow, I., and Emes, M. 2007. Investigation of digestibility in vitro and physicochemical properties of A- and B-type starch from soft and hard wheat flour. Cereal Chemistry **84**(1): 15-21.

Maghirang, E.B., Lookhart, G.L, Bean, S.R., Pierce, R.O, Xie, F., Caley, M.S., Wilson, J.D., Seabourn, B.W., Ram, M.S., Park, S.H., Chung, O.K. and Dowell, F.E. 2006. Comparison of quality characteristics and breadmaking functionality of hard red winter and hard red spring wheat. Cereal Chemistry **83**(5): 520-528

Mares, D.J., and Stone, B.A. 1973. Studies on wheat endosperm. 1. Chemical composition and ultrastructure of cell-walls. Australian Journal of Biological Sciences **26**(4): 793-812.

Matser, A.M., and Steeneken, P.A.M. 1998. Origins of the poor filtration characteristics of wheat starch hydrolysates. Cereal Chemistry **75**(3): 289-293.

McLaughlin, S.B., and Walsh, M.E. 1998. Evaluating environmental consequences of producing herbaceous crops for bioenergy. Biomass & Bioenergy **14**(4): 317-324.

Meredith, P. 1981. Large and Small Starch Granules in Wheat - Are They Really Different. Starke **33**(2): 40-44.

Montesinos, T., and Navarro, J.M. 2000a. Production of alcohol from raw wheat flour by Amyloglucosidase and Saccharomyces cerevisiae. Enzyme and Microbial Technology **27**(6): 362-370.

Montesinos, T., and Navarro, J.M. 2000b. Role of the maltose in the simultaneous-saccharification-fermentation process from raw wheat starch and Saccharomyces cerevisiae. Bioprocess Engineering **23**(4): 319-322.

Moorthy, S.N. 2002. Physicochemical and functional properties of tropical tuber starches: A review. Starch-Starke **54**(12): 559-592.

Morrison, W.R., and Gadan, H. 1987. The amylose and lipid contents of starch granules in developing wheat endosperm Journal of Cereal Science **5**(3): 263-275.

Moxley, G., Zhu, Z.G., and Zhang, Y.H.P. 2008. Efficient sugar release by the cellulose solvent-based lignocellulose fractionation technology and enzymatic cellulose hydrolysis. Journal of Agricultural and Food Chemistry **56**(17): 7885-7890.

MRA. 2008. World's ethanol production forecast 2008-2012. Market Research Analyst. http://www.marketresearchanalyst.com/2008/01/26/world-ethanol-production-forecast-2008-2012/

MSGA. 2007. Seed Manitoba 2007. Manitoba Seed Growers Association, http://www.seedmb.ca/.

NAS. 2003. National Academy of Sciences: Frontiers in Agricultural Research: Food, Health, Environment and Communities, http://dels.nas.edu/rpt_briefs/frontiers_in_ag_final%20for%20print.pdf.

Needleman, R. 1991. Control of maltase synthesis of yeast Molecular Microbiology **5**(9): 2079-2084.

Noda, T., Kimura, T., Otani, M., Ideta, O., Shimada, T., Saito, A., and Suda, I. 2002. Physicochemical properties of amylose-free starch from transgenic sweet potato. Carbohydrate Polymers **49**(3): 253-260.

Ochoa, S., Yoo, A., Repke, J.U., Wozny, G., and Yang, D.R. 2007. Modeling and parameter identification of the simultaneous saccharification-fermentation process for ethanol production. Biotechnology Progress **23**(6): 1454-1462.

Otterstedt, K., Larsson, C., Bill, R.M., Stahlberg, A., Boles, E., Hohmann, S., and Gustafsson, L. 2004. Switching the mode of metabolism in the yeast Saccharomyces cerevisiae. Embo Reports **5**(5): 532-537. doi:10.1038/sj.embor.7400132.

Pampulha, M.E., and Loureirodias, M.C. 1990. Activity of glycolytic-enzymes of Saccharomyces-cerevisiae in teh presence of acetic-acid. Applied Microbiology and Biotechnology **34**(3): 375-380.

Panchal, C.J., and Stewart, G.G. 1979. Utilization of wort carbohydrates. Brewers Digest **54**(6): 36-&.

Park, J.T., and Rollings, J.E. 1994. Effects of substrate branching characteristics on kinetics of enzymatic depolymerization of mixed linear and branched polysaccharides. 1. Amylose/amylpectin alpha-amylolysis. Biotechnology and Bioengineering **44**(7): 792-800.

Park, S.H., Wilson, J.D., Chung, O.K., and Seib, P.A. 2004. Size distribution and properties of wheat starch granules in relation to crumb grain score of pup-loaf bread. Cereal Chemistry **81**(6): 699-704.

Park, S.H., Wilson, J.D., and Seabourn, B.W. 2009. Starch granule size distribution of hard red winter and hard red spring wheat: Its effects on mixing and breadmaking quality. Journal of Cereal Science **49**(1): 98-105.

Patzek, T.W. 2004. Thermodynamics of the corn-ethanol biofuel cycle. Critical Reviews in Plant Sciences **23**(6): 519-567.

Peng, M., Gao, M., Abdel-Aal, E.S.M., Hucl, P., and Chibbar, R.N. 1999. Separation and characterization of A- and B-type starch granules in wheat endosperm. Cereal Chemistry **76**(3): 375-379.

Phelps, S.M., Beres, B., Johnson, E., Pozniak, C., and Chant, S. 2009. 2008 Western Canadian Ethanol Feedstock Trial Interim Report - Feb 10, 2009. Western Applied Research Corporation, www.warc.ca/reports/2008_Western_Canadian_Ethanol_Feedstock_Trial_62.pdf.

Pimentel, D., Marklein, A., Toth, M.A., Karpoff, M.N., Paul, G.S., McCormack, R., Kyriazis, J., and Krueger, T. 2009. Food Versus Biofuels: Environmental and Economic Costs. Human Ecology **37**(1): 1-12.

Pryor, F.L. 2009. The Economics of Gasohol. Contemporary Economic Policy **27**(4): 523-537.

Quadrelli, R., and Peterson, S. 2007. The energy-climate challenge: Recent trends in CO2 emissions from fuel combustion. Energy Policy **35**(11): 5938-5952.

Raeker, M.O., Gaines, C.S., Finney, P.L., and Donelson, T. 1998. Granule size distribution and chemical composition of starches from 12 soft wheat cultivars. Cereal Chemistry **75**(5): 721-728.

Rajagopal, D., Sexton, S.E., Roland-Holst, D., and Zilberman, D. 2007. Challenge of biofuel: filling the tank without emptying the stomach? Environmental Research Letters **2**(4).

Rakszegi, M., Boros, D., Kuti, C., Lang, L., Bedo, Z., and Shewry, P.R. 2008. Composition and End-Use Quality of 150 Wheat Lines Selected for the HEALTHGRAIN Diversity Screen. Journal of Agricultural and Food Chemistry **56**(21): 9750-9757.

RFA. 2008. FO Lichts Ethanol Production Costs: A Worldwide Survery, a special study from FO Lichts and Agra CEAS Consulting. Renewable Fuels Association, http://www.agra-net.com/portal2/home.jsp?template=productpage&pubid=ag090.

Ring, S.G., and Selvendran, R.R. 1980. Isolation and analysis of cell-wall material from beeswing wheat bran (Triticum aestivum). Phytochemistry **19**(8): 1723-1730.

Rooney, L.W., and Pflugfelder, R.L. 1986. Factors Affecting Starch Digestibility with Special Emphasis on Sorghum and Corn. Journal of Animal Science **63**(5): 1607-1623.

Sanchez, O.J., and Cardona, C.A. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresource Technology **99**(13): 5270-5295.

Sasaki, T., Yasui, T., Matsuki, J., and Satake, T. 2002. Comparison of physical properties of wheat starch gels with different amylose content. Cereal Chemistry **79**(6): 861-866.

Searchinger, T., Heimlich, R., Houghton, R.A., Dong, F.X., Elobeid, A., Fabiosa, J., Tokgoz, S., Hayes, D., and Yu, T.H. 2008. Use of US croplands for biofuels increases greenhouse gases through emissions from land-use change. Science **319**(5867): 1238-1240.

SeCan. 2006. Technical Bulletin: AC Andrew (SWS 241) Soft White Spring Wheat. http://www.terragrainfuels.com/graininfo/AC-Andrew-Nov-2006.pdf.

Sims, R. 2008. From 1st to 2nd Generation Biofuel Technologies: An overview of current industry and RD&D activities. International Energy Agency Bioenergy, http://www.iea.org/publications/free_new_Desc.asp?PUBS_ID=2079.

Singh, N., Singh, S., Isono, N., Noda, T., and Singh, A.M. 2009. Diversity in amylopectin structure, thermal and pasting properties of starches from wheat varieties/lines. International Journal of Biological Macromolecules **45**(3): 298-304.

Slaughter, D.C., Norris, K.H., and Hruschka, W.R. 1992. Quality and classification of hard red wheat Cereal Chemistry **69**(4): 428-432.

Slingerland, S., and van Geuns, L. 2005. Clingendael International Energy Program ; Drivers for an international biofuels market, Discussion Paper. Future Fuel Seminar, http://www.clingendael.nl.

Smith, T.C., Kindred, D.R., Brosnan, J.M., Weightman, R.M., Shepherd, M., and Sylvestery-Bradley, R. 2006. Wheat as feedstock for alcohol production. Home Grown Cereals Authority Research Review **2006**(61).

Song, Y., and Jane, J. 2000. Characterization of barley starches of waxy, normal, and high amylose varieties. Carbohydrate Polymers **41**(4): 365-377.

Sosulski, K., and Sosulski, F. 1994. Wheat as a feedstock for fuel ethanol. Applied Biochemistry and Biotechnology **45**(6): 169-180.

Sosulski, K., Wang, S.M., Ingledew, W.M., Sosulski, F.W., and Tang, J.M. 1997. Preprocessed barley, rye, and Triticale as a feedstock for an integrated fuel ethanol feedlot plant. Applied Biochemistry and Biotechnology **63-65**(1): 59-70.

Soulaka, A.B., and Morrison, W.R. 1985. The amylose and lipid contents, dimensions, and gelatinization characteristics of some wheat starches and their A-granul and B-granule fractions Journal of the Science of Food and Agriculture **36**(8): 709-718.

Souza, E.J., Martin, J.M., Guttieri, M.J., O'Brien, K.M., Habernicht, D.K., Lanning, S.P., McLean, R., Carlson, G.R., and Talbert, L.E. 2004. Influence of genotype, environment, and nitrogen management on spring wheat quality. Crop Science **44**(2): 425-432.

Srinivasan, S. 2009. The food v. fuel debate: A nuanced view of incentive structures. Renewable Energy **34**(4): 950-954.

Stephanopoulos, G. 2008. Metabolic engineering: Enabling technology for biofuels production. Metabolic Engineering **10**(6): 293-294.

Stoddard, F.L. 1999. Survey of starch particle-size distribution in wheat and related species. Cereal Chemistry **76**(1): 145-149.

Swanston, J.S., Newton, A.C., Brosnan, J.M., Fotheringham, A., and Glasgow, E. 2005. Determining the spirit yield of wheat varieties and variety mixtures. Journal of Cereal Science **42**(1): 127-134.

Swanston, J.S., Smith, P.L., Gillespie, T.L., Brosnan, J.M., Bringhurst, T.A., and Agu, R.C. 2007. Associations between grain characteristics and alcohol yield among soft wheat varieties. Journal of the Science of Food and Agriculture **87**(4): 676-683.

Swinkels, J.J.M. 1985. Composition and properties of commercial native starches Starke **37**(1): 1-5.

Tan, K.T., Lee, K.T., and Mohamed, A.R. 2008. Role of energy policy in renewable energy accomplishment: The case of second-generation bioethanol. Energy Policy **36**(9): 3360-3365. doi:10.1016/j.enpol.2008.05.016.

Tao, L., and Aden, A. 2009. The economics of current and future biofuels. In Vitro Cellular & Developmental Biology-Plant **45**(3): 199-217.

Tester, R.F., Karkalas, J., and Qi, X. 2004a. Starch - composition, fine structure and architecture. Journal of Cereal Science **39**(2): 151-165.

Tester, R.F., Karkalas, J., and Qi, X. 2004b. Starch structure and digestibility enzyme-substrate relationship. World's Poultry Science Journal **60**(2): 186-195.

Tester, R.F., and Morrison, W.R. 1990. Swelling and gelatinization of cereal starches. 1. Effects of amylopectin, amylose, and lipids Cereal Chemistry **67**(6): 551-557.

TGF. 2010. Terra Grain Fuels - Grow With Us, http://www.terragrainfuels.com/grain_info.php.

Thomas, K.C., and Ingledew, W.M. 1990. Fuel alcohol production - effects of free amino nitrogen on fermentation of very high gravity wheat mashes Applied and Environmental Microbiology **56**(7): 2046-2050.

Trenberth, K.E., P.D. Jones, P. Ambenje, R. Bojariu, D. Easterling, A. Klein Tank, D. Parker, F. Rahimzadeh, J.A. Renwick M. Rusticucci, B. Soden and P. Zhai. 2007. Observations: Surface and Atmopheric Climate Change. *In* Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. *Edited by* S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L Miller. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

USDA. 2007. Major Land Uses. United States Department of Agriculture Economic Research Services http://www.ers.usda.gov/data/majorlanduses.

USDA. 2008. National Agricultural Statistics Service - Corn, Field United States Department of Agriculture http://www.nass.usda.gov/QuickStats/index2.jsp.

USDA. 2009. Agricultural Research Services. United States Department of Agriculture, http://www.ars.usda.gov/main/main.html.

Van Hung, P., Maeda, T., and Morita, N. 2007. Study on physicochemical characteristics of waxy and high-amylose wheat starches in comparison with normal wheat starch. Starch-Starke **59**(3-4): 125-131.

van Maris, A.J.A., Abbott, D.A., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M.A.H., Wisselink, H.W., Scheffers, W.A., van Dijken, J.P., and Pronk, J.T. 2006. Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. *In conference proceedings: International Symposium on full-circle- microbial cycling of the elements*. Antoine Van Leeuwenhoek Intenational Journal of General and Molecular Microbiology **90**(4):391-418.

Wang, P., Singh, V., Xue, H., Johnston, D.B., Rausch, K.D., and Tumbleson, M.E. 2007. Comparison of raw starch hydrolyzing enzyme with conventional liquefaction and saccharification enzymes in dry-grind corn processing. Cereal Chemistry **84**(1): 10-14.

Wang, S., Thomas, K.C., Ingledew, W.M., Sosulski, K., and Sosulski, F.W. 1997. Rye and triticale as feedstock for fuel ethanol production. Cereal Chemistry **74**(5): 621-625.

Wilson, J.D., Bechtel, D.B., Todd, T.C., and Seib, P.A. 2006. Measurement of wheat starch granule size distribution using image analysis and laser diffraction technology. Cereal Chemistry **83**(3): 259-268.

Wu, X., Zhao, R., Bean, S.R., Seib, P.A., McLaren, J.S., Madl, R.L., Tuinstra, M., Lenz, M.C., and Wang, D. 2007. Factors impacting ethanol production from grain sorghum in the dry-grind process. Cereal Chemistry **84**(2): 130-136.

Wu, X., Zhao, R., Wang, D., Bean, S.R., Seib, P.A., Tuinstra, M.R., Campbell, M., and O'Brien, A. 2006. Effects of amylose, corn protein, and corn fiber contents on production of ethanol from starch-rich media. Cereal Chemistry **83**(5): 569-575.

Yasui, T., Sasaki, T., and Matsuki, J. 2005. Variation in the chain-length distribution profiles of endosperm starch from Triticum and Aegilops. species. Starch-Starke **57**(11): 521-530.

Yonsel, S., Bulbul-Caliskan, G., Koni, M., and Dagasan, L. 2007. Monitoring of yeast metabolism with calorimetry. Chemical and Biochemical Engineering Quarterly **21**(4): 395-408.

Zastrow, C.R., Mattos, M.A., Hollatz, C., and Stambuk, B.U. 2000. Maltotriose metabolism by Saccharomyces cerevisiae. Biotechnology Letters **22**(6): 455-459.

Zeng, M., Morris, C.F., Batey, I.L., and Wrigley, C.W. 1997. Sources of variation for starch gelatinization, pasting, and gelation properties in wheat. Cereal Chemistry **74**(1): 63-71.

Zhang, G., Ao, Z., and Hamaker, B.R. 2008a. Nutritional property of endosperm starches from maize mutants: A parabolic relationship between slowly digestible starch and

amylopectin fine structure. Journal of Agricultural and Food Chemistry **56**(12): 4686-4694.

Zhang, G.Y., Sofyan, M., and Hamaker, B.R. 2008b. Slowly digestible state of starch: Mechanism of slow digestion property of gelatinized maize starch. Journal of Agricultural and Food Chemistry **56**(12): 4695-4702.

Zhang, T., and Oates, C.G. 1999. Relationship between alpha-amylase degradation and physico-chemical properties of sweet potato starches. Food Chemistry **65**(2): 157-163.

Zhao, R., Wu, X., Seabourn, B.W., Bean, S.R., Guan, L., Shi, Y.C., Wilson, J.D., Madl, R., and Wang, D. 2009. Comparison of Waxy vs. Nonwaxy Wheats in Fuel Ethanol Fermentation. Cereal Chemistry **86**(2): 145-156.

Zheng, X.H., Damore, T., Russell, I., and Stewart, G.G. 1994. Transport kinetics of maltotriose in strains of Saccharomyces Journal of Industrial Microbiology **13**(3): 159-166.

Zobel, H.F., Young, S.N., and Rocca, L.A. 1988. Starch gelatinization - an X-Ray diffraction study Cereal Chemistry **65**(6): 443-446.

Appendix A: Sample Calculations

Calculation A.1: Theoretical ethanol and carbon dioxide yield calculation from glucose during fermentation.

Reaction A.1 Stoichiometric Relationship of Glucose to Ethanol and Carbon Dioxide

$$\underset{\text{(Glucose)}}{C_6H_{12}O_6} \text{ ----------> } \underset{\text{(Ethanol)}}{2\ C_2H_5OH} + \underset{\text{(Carbon Dioxide)}}{2\ CO_2}$$

Molecular weights (MW) of compounds:

Glucose = 180.16 g/mole
Ethanol = 46.05 g/mole
Carbon Dioxide = 44 g/mole

Ethanol Yield Calculation:

*Assuming 1g Glucose starting material

1g Glucose x (180.16 g-Glucose/mole)$^{-1}$ x (2 moles Ethanol/1 mole Glucose)
= 0.011 moles Ethanol

0.011 moles Ethanol x (46.05 g-Ethanol/mole) = 0.511 g-Ethanol/ g-Glucose
= 51.1 % (w/w)

CO_2 Yield Calculation:

1g Glucose x (180.16 g-Glucose/mole)$^{-1}$ x (2 moles CO_2/1 mole Glucose)
= 0.011 moles CO_2

0.011 moles Carbon Dioxide x (44 g/mole)= 0.488 g-CO_2/ g-Glucose
= 48.8 % (w/w)

Therefore 1 g/L of glucose in the initial solution upon fermentation should theoretically yield 0.51 g/L of ethanol and 0.49 g/L of CO_2 (0.25 L of gaseous CO_2 at STP).

Calculation A.2: Calculated ethanol yield per tonne of whole grain.

Sample calculation for S700PR

Experimentally determined values:

Ethanol yield on pure starch: 0.500 g/g-starch

Total starch content in kernel: 60.61%

Weight per kernel: 35.59 mg

Constants:

$\rho_{ethanol}$ = 789 g/L

Liters of ethanol per kg-starch:

(0.500 g/g-starch) x $(789\ g/L)^{-1}$ x 1000g/kg = 0.633 L-ethanol/kg-starch

Starch content per kernel:

0.6061 x 35.59 mg x 1g/1000mg x 1kg/1000g = 2.61×10^{-5} kg-starch/kernel

Number of kernels per tonne of grain:

$(35.59 mg/kernel)^{-1}$ x 1kg/1×10^{6}mg x 1000kg/tonne = 2.81×10^{7} kernels/tonne

Ethanol per tonne:

0.633 L-ethanol/kg-starch x 2.61×10^{-5} kg-starch/kernel x 2.81×10^{7} kernels/tonne

= 384.06 L-ethanol/tonne

Appendix B: Amylopectin Fine Structure

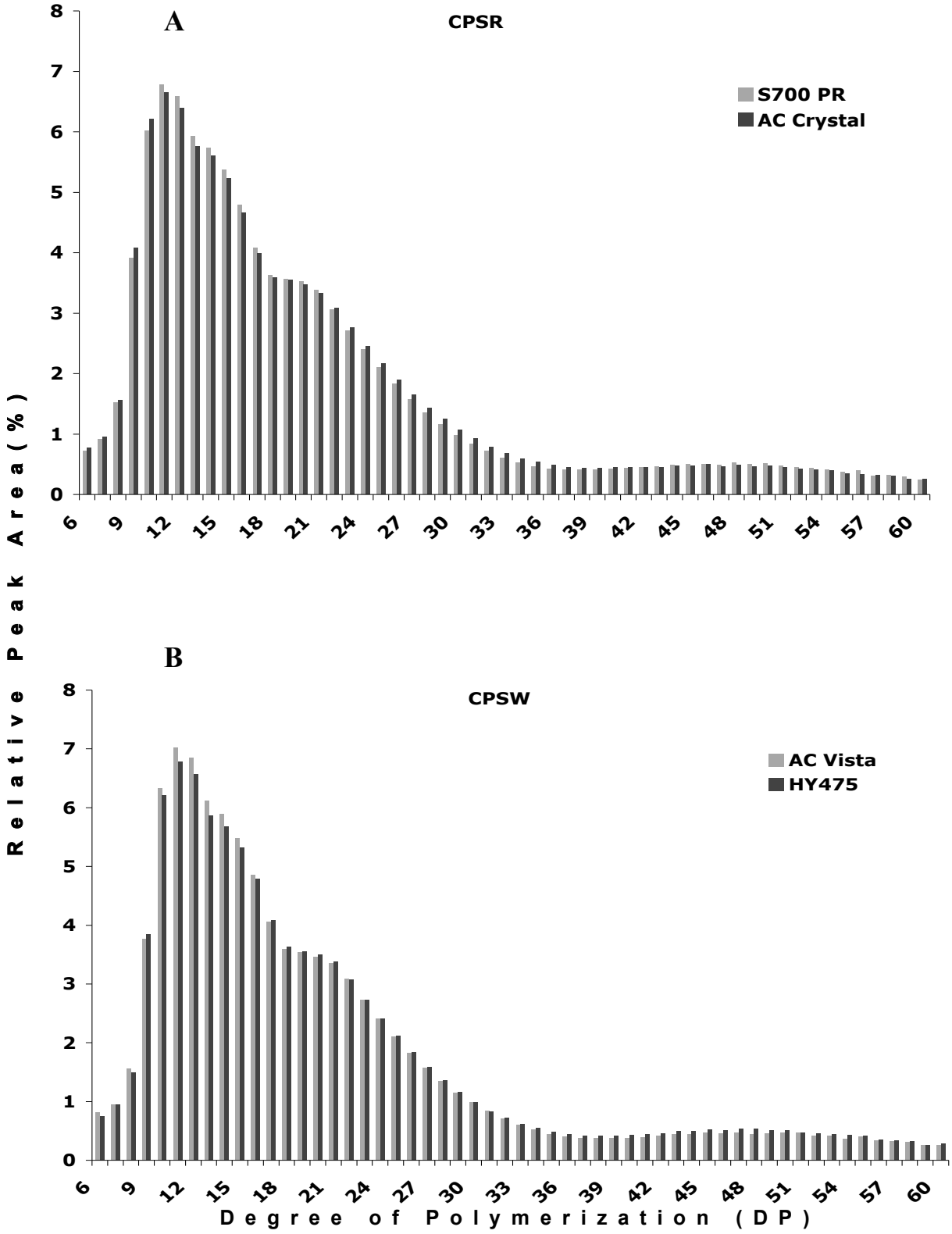

Figure B.1. Normalized branch chain length distribution of amylopectin for wheat and triticale cultivars analyzed by HPAEC-PAD. A) CPSR: AC Crystal, S700PR B) CPSW: AC Vista, HY 475

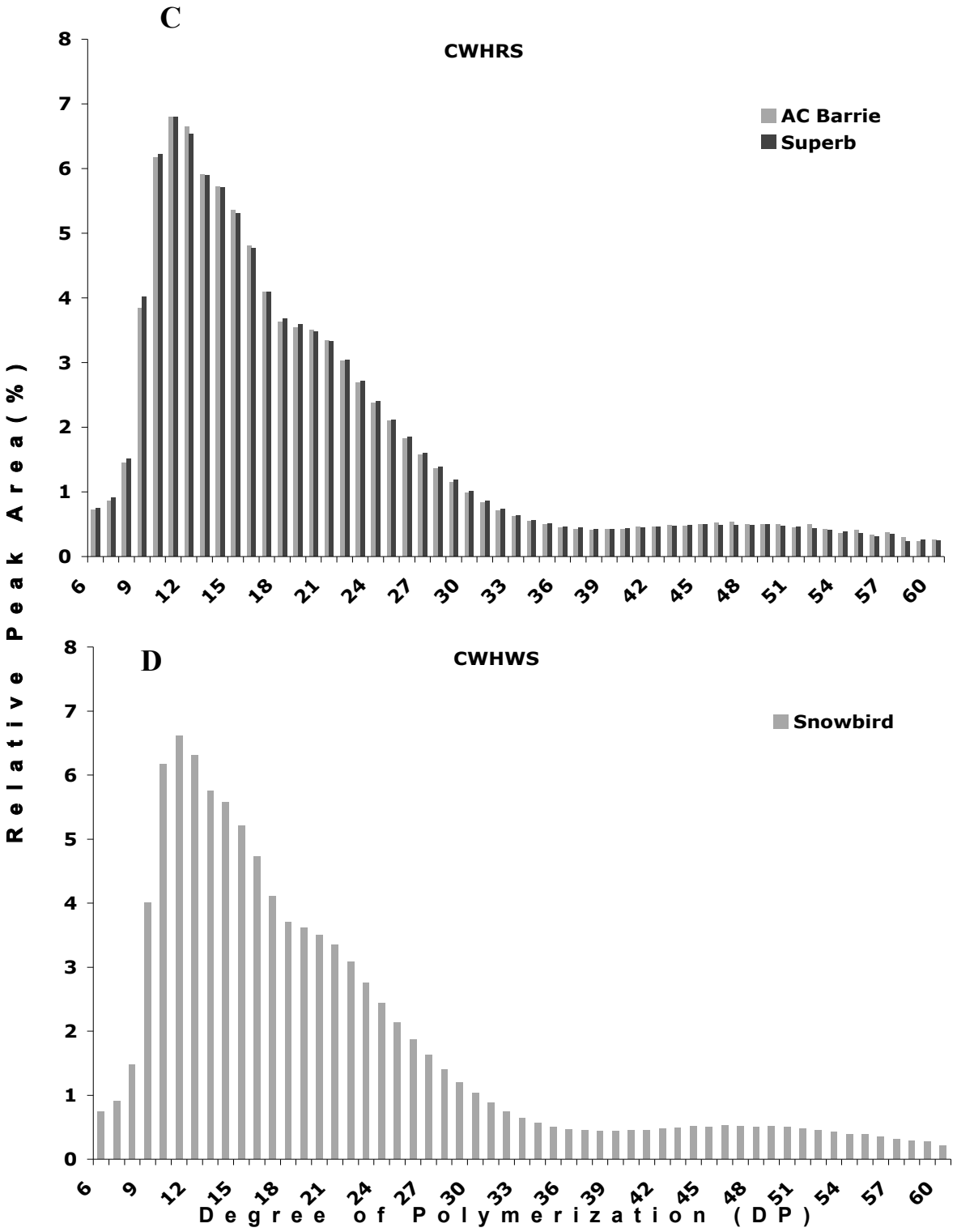

Figure B.1. (cont) Normalized branch chain length distribution of amylopectin for wheat and triticale cultivars analyzed by HPAEC-PAD. C) CWRS: AC Barrie, Superb, D) CWHWS: Snowbird

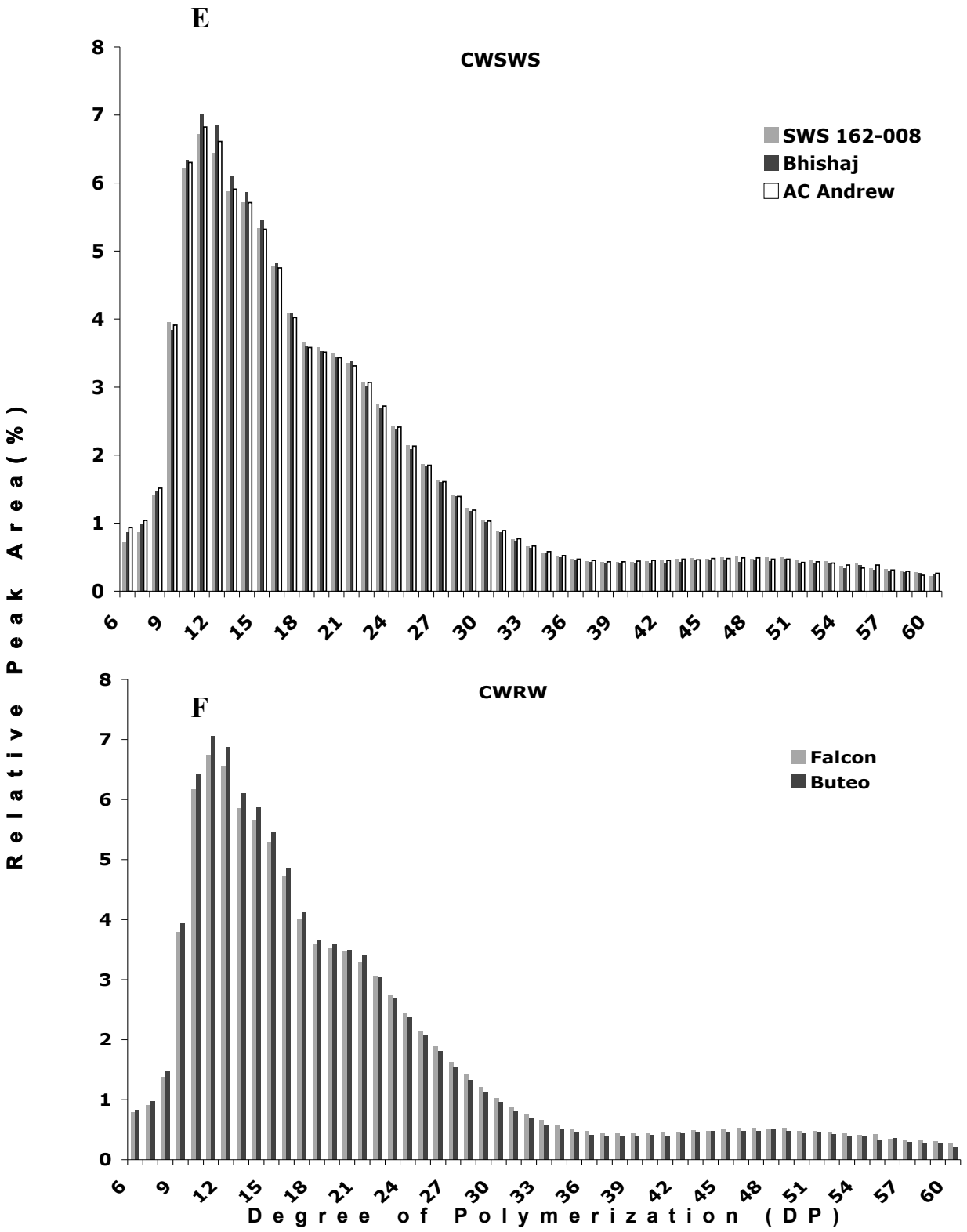

Figure B.1. (cont) Normalized branch chain length distribution of amylopectin for wheat and triticale cultivars analyzed by HPAEC-PAD. E) CWSWS: SWS 162-008, Bhishaj, AC Andrew F) CWRW: CDC Falcon, Buteo

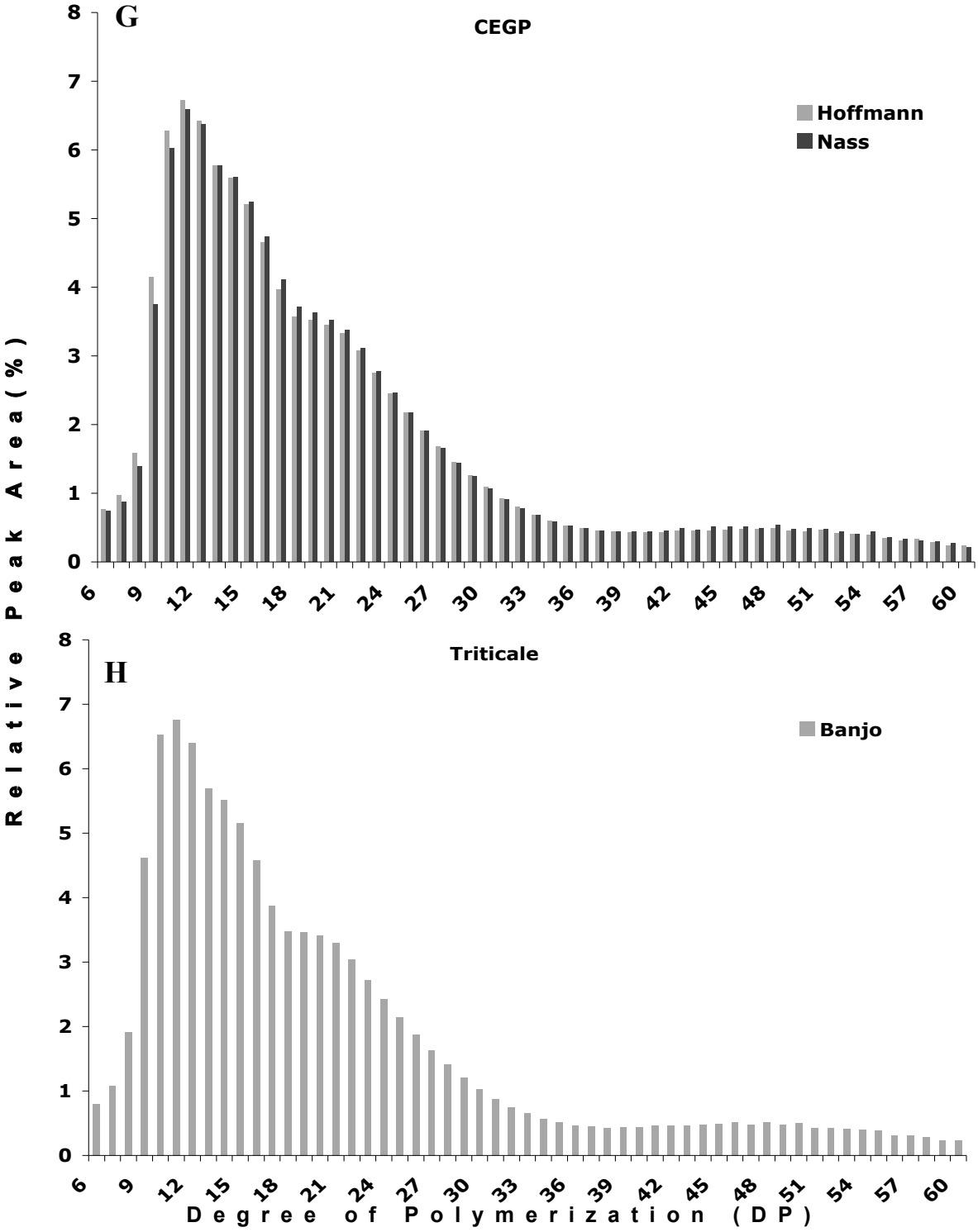

Figure B.1. (cont) Normalized branch chain length distribution of amylopectin for wheat and triticale cultivars analyzed by HPAEC-PAD. G) CEGP: Hoffmann, Nass H) Triticale: Banjo.